Stone clearance rate with *Phyllanthus niruri* (Chanca Piedra)

The following studies observed the effect of *P. niruri* on stone-free rate, reduction in stone size/number, or their radiologic clearance.

ORIGINAL ARTICLE

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Effect of *phyllanthus niruri* on metabolic parameters of patients with kidney stone: a perspective for disease prevention

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ABSTRACT

*Phyllanthus niruri (P.niruri) o*r stone breaker is a plant commonly used to reduce stone risk, however, clinical studies on this issue are lacking.
Objective: To prospectively evaluate the effect of *P. niruri* on the urinary metabolic parameters of patients with urinary lithiasis.
Materials and Methods: We studied 56 patients with kidney stones <10mm. Clinical, metabolic, and ultrasonography assessment was conducted before (baseline) the use of *P. niruri* infusion for 12-weeks *(P. niruri)* and after a 12-week (wash out) Statistical analysis included ANOVA for repeated measures and Tukey's/McNemar´s test for categorical variables. Significance was set at 5%.
Results: Mean age was 44±9.2 and BMI was 27.2±4.4kg/m2. Thirty-six patients (64%) were women. There were no significant changes in all periods for anthropometric and several serum measurements, including total blood count, creatinine, uric acid, sodium, potassium, calcium, urine volume and pH; a significant increase in urinary potassium from 50.5±20.4 to 56.2±21.8 mg/24-hour (p=0.017); magnesium/creatinine ratio 58±22.5 to 69.1±28.6mg/gCr24-hour (p=0.013) and potassium/creatinine ratio 39.3±15.1 to 51.3±34.7mg/gCr24-hour (p=0.008) from baseline to wash out. The kidney stones decreased from 3.2±2 to 2.0±2per patient (p<0.001). In hyperoxaluria patients, urinary oxalate reduced from 59.0±11.7 to 28.8±16.0mg/24-hour (p=0.0002), and in hyperuricosuria there was a decrease in urinary uric acid from 0.77±0.22 to 0.54±0.07mg/24-hour (p=0.0057).
Conclusions: P.niruri intake is safe and does not cause significant adverse effects on serum metabolic parameters. It increases urinary excretion of magnesium and potassium caused a significant decrease in urinary oxalate and uric acid in patients with hyperoxaluria and hyperuricosuria. The consumption of *P.niruri* contributed to the elimination of urinary calculi.

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INTRODUCTION

Risk factors for urolithiasis include congenital, genetic, environmental, dietary and metabolic aspects; chronic diseases including obesity, hypertension and diabetes are also associated with urinary calculus formation (1). Overall, urinary

calculi derive from a combination of some of the factors involved in its pathophysiology (1). The worldwide prevalence of urinary calculi is 8.8% and is more frequent in Caucasians, obese individuals, and those with a low income (1). The recurrence rate of urinary calculi is 50% within 10 years of the first episode (2). In addition to the conditions stated above, metabolic disorders such as hypercalciuria and hypocitraturia are typically involved in the genesis of urinary calculi (3).

Knowledge of the pathophysiological mechanisms involved and their risk factors, such as low urinary volume and high intake of calories, sodium and protein are important for modifying the natural history of the disease (2). Indeed, a reduction in the recurrence of urinary calculi of at least 50% can be achieved with dietary guidelines, lifestyle changes and the use of specific medications (4, 5).

In addition to conventional treatment for lithiasis, medicinal plants have long been used worldwide (6). *Phyllanthus niruri* or "stone breaker tea" is one such natural alternative that is inexpensive, easy to obtain and has a low incidence of adverse effects (7). To date, anti-inflammatory (8), anti-hyperuricemic (9), and diuretic properties have been described for this plant (10). Although many studies have shown the beneficial effects of *P. niruri* and its potential to inhibit the formation of kidney stones, clinical studies remain scarce (11, 12).

Thus, the aim of this study was to evaluate the effects of *P. niruri* on metabolic parameters in patients with urolithiasis.

This protocol was submitted to and approved by the Ethics Committee for Research Project Analysis of the Clinical Hospital, University of São Paulo, Medical School under number 0304/11. It was also approved and sponsored by the Foundation of Research Support of São Paulo (FAPESP) under number 12/50031-7.

MATERIALS AND METHODS

The study was developed at the Urologic Division of the Clinical Hospital, University of São Paulo, Medical School. All patients included presented one or multiple stones smaller than 10mm. Diagnosis was based on ultrasonography or computed tomography (CT). The age of the patients ranged between 18 and 60 years.

Exclusion criteria included patients with a serum creatinine level >2.0mg/dL, urinary tract infection, non-controlled diabetes, chronic liver disease, cancer or pregnant women.

The study was divided into three stages: baseline, *P. niruri* and washout. The baseline stage was that prior to intervention. The *P. niruri* stage consisted of 12 weeks of ingestion of an infusion tea prepared with the dry extract of *P. niruri*, according to literature recommendations (13), a week of rest was included without using the plant after each week of consumption (2 weeks). The final step, called washout, involved 12 weeks without ingestion of *P. niruri*. The patients themselves were the controls, and each patient was followed for 26 weeks. The patients were subjected to clinical, anthropometric, serum and urinary metabolic analyses at all stages of the study.

Demographic data included age, race, sex, family history and medication use. Clinical data comprised systolic and diastolic blood pressure and anthropometric evaluation (weight, height, body mass index (BMJ). Serum biochemical and urinary analyses and renal ultrasonography were performed at baseline, immediately after *P. niruri* use and at the end of the washout period. Image evaluation was performed for all patients by the same radiology physicians who were blinded to the group composition.

Serum biochemical analysis was performed during the baseline, *P. niruri* and washout periods and included a complete blood count and assessment of urea, creatinine, sodium, potassium, glucose (fasting), uric acid, total and ionized calcium, beta human chorionic gonadotropin (HCG; females only), total cholesterol and fractions, triglycerides, alanine aminotransferase, aspartate aminotransferase, gamma-glutamyl transpeptidase, amylase and bilirubin levels. Serum samples were collected from all participants after fasting for 12 hours.

Twenty-four-hour urinary measurements included calcium, oxalate, citrate, uric acid, magnesium, sodium, potassium, creatinine, urea and phosphorus levels. Urinalysis with urinary pH

measurement and a urine culture was performed using spontaneous voided urine.

The criteria for evaluation of metabolic abnormalities in 24-hour urine samples (mg/g creatinine) were hypercalciuria (calcium >240/>200mg/24-hour), hyperoxaluria (oxalate>40mg/24-hour), hypocitraturia (citrate<320/<290mg/24-hour), hypernatriuria (sodium >150mg/24-hour) hyperuricosuria (uric acid >0.75/>0.6g/24-hour), abnormal pH (<5.8 and >6.2) and low urine volume (<2L/day) for men and women, respectively.(4).

After receiving clinical, laboratory and abdominal ultrasound evaluations, the patients included in the study received a monthly visit and 60 sachets of *P. niruri* as a dry extract containing 4.5g of the herb. The patients were instructed to prepare an infusion containing 250mL of boiling water for each 4.5g herb sachet and to drink two sachets/day.

Extracts of *P. niruri* (family Euphorbiaceae) were obtained from qualified manufacturers and from medicinal plant laboratories, and we obtained physicochemical and microbiological analysis reports for all lots used in this study. The plant was produced in Brazil, subjected to a shade drying method, stored at a temperature of 15-35°C in a dry place and conserved with gamma irradiation to avoid contamination by fungi or bacteria. The minimum tannin content was 1.5%, and the moisture content was 17%. Indications for use in disorders of the urinary tract include renal lithiasis, cramps, cystitis and nephritis. The plant is known to have analgesic, anti-inflammatory and hepatoprotective properties.

The use of medications for hypertension or other diseases was not discontinued for the study or considered in the analysis. Only patients using potassium citrate were instructed to discontinue its use two months prior to initiation of the study to avoid interference with the results of 24-hour urine citrate dosing.

Statistical analysis was performed with SAS-Statistical Analysis Software Version 9. Continuous variables are presented as the mean and standard deviation, and categorical variables are presented as frequency percentages. We used analysis of variance (ANOVA) for repeated measures, which takes into account patients being evaluated at three different times (repeated measures) for comparing the results. Significant differences between periods or stages of evaluation were compared with Tukey's test and the McNemar test. The significance level was set at 5%.

RESULTS

In total, 430 patients were enrolled and initially screened, and 75 patients were considered eligible according to the inclusion criteria. Fifty-six patients completed the study after a few dropped out due to work or personal problems, surgeries or other treatments for non-urological (breast carcinoma, biliary calculus) diseases.

Of the 56 patients who remained, 36 (64.3%) were women, and 52 (92.8%) were Caucasians. The mean age was 44±9.2 years (22-58 years), and the baseline BMI was 27.2±4.4kg/m^2. Thirty patients (53.6%) had a family history of calculi, 53 (94.6%) were sedentary, 27 (48.2%) had hypertension, and 26 (46.4%) had metabolic syndrome.

Of the studied cohort, 26 patients (46.4%) were using antihypertensive drugs; one patient (1.8%) was under hypoglycaemic medication, and four patients (7.1%) were using antidepressants, among other medications. Diastolic blood pressure showed a slight increase, though not significant, during the *P. niruri* period, reaching 76.0±10.5mmHg and then decreasing to 72.5±10.5mmHg (p=0.02) during the washout period.

Abdominal pain was reported by 37 (66.1%) patients during the *P. niruri* period; dysuria occurred in 11 (19.6%) cases, haematuria in eight (14.3%) cases, and nausea and epigastric pain in six cases each (10.7%). However, these symptoms did not lead to discontinuation of infusion use in any case.

No significant changes in serum measurements, except for a significant decrease in alkaline phosphatase after the use of *P. niruri* when compared to the baseline period (67.7±22.2x63.5±20.6mg/dL; p=0.017), were found.

The 24-hour urine analysis revealed only a significant increase in potassium levels between the basal and washout periods, from 47.3±16.7mg/24-

-hour to 56.2±21.8mg/24-hour (p=0.017). However, a trend towards increased urinary potassium was noted during the use of the tea.

Analysis of electrolytes adjusted for 24-hour urine creatinine (Cr) revealed an increase in potassium and magnesium levels. This increase reached statistical significance in the washout period, as summarized in Table-1.

In the baseline stage, hypernatriuria was the most frequent urinary disturbance, occurring in 34 patients (60.7%). Hypocitraturia and hypercalciuria were observed in 24 (42.8%) cases, hyperuricosuria in six (10.7%), hyperoxaluria in five (8.9%), and low urine volume in 31 (55.3%). Furthermore, the pH value changed in 21 (37.5%) patients.

Evaluation of patients with urinary abnormalities at the baseline stage showed a tendency towards an increase in urinary citrate among the hypocitraturic patients (211.8±123.7 to 322.3±145.8mg/24-hour, p=0.2193). In addition, oxalate was significantly reduced, from 59.0±11.7 to 28.8±16.0mg/24-hour (p=0.0002), among those with hyperoxaluria, and uric acid was significantly decreased, from 0.77±0.22 to 0.54±0.07mg/24--hour (p=0.0057), among hyperuricosuric patients (Table-2).

Ultrasonography evaluation performed in the three periods of the study revealed that the total number of stones decreased in 38 (67.8%) patients, as summarized in Table-3 (from 3.2±2.02 to 2.0±2.07) and size. In 10 patients (17.8%), no alterations in the number of calculi were noted, and in eight patients (14.3%), an increase in the number of upper urinary stones was observed after the *P. niruri* period. Some patients reported spontaneous stone passage between the 21st and 70th day of the *P. niruri* period: four patients eliminated six stones, and another five individuals reported the presence of sandy fragments in the urine during the *P. niruri* period.

DISCUSSION

The formation of urinary calculi is associated with different risk factors. Its prevalence is

Table 1 - Urinary parameters in patients treated with *P. niruri*.

Measure	MEAN (SD)			ANOVA P	TUKEY		
	Baseline	mg/vol.24-hour *P. niruri*	Wash out		Baseline x *P. niruri*	Baseline x Wash out	*P. niruri* x Wash out
Calcium	202.7±116.1	211.8±96.2	190.3±92.6	0.401	0.849	0.675	0.371
Uric acid	0.5±0.2	0.5±0.2	0.5±0.2	0.875	0.870	0.990	0.934
Sodium	171.6±74.0	166.1±78.9	182.7±62.2	0.368	0.881	0.610	0.344
Urea	20.1±6.0	18.6±7.7	20.9±8.4	0.070	0.278	0.679	0.063
Potassium	**47.3±16.7**	**50.5±20.4**	**56.2±21.8**	**0.023**	0.536	**0.017**	0.192
Citrate	379.9±191.1	398.1±219.0	412.8±175.8	0.515	0.922	0.488	0.739
Oxalate	24.4±15.5	23.9±14.5	22.3±9.7	0.639	0.952	0.619	0.795
Creatinine	1.3±0.5	1.3±0.5	1.2±0.5	0.672	0.682	0.773	0.989
Phosphorus	755.3±308.8	792.4±324.6	684.9±281.1	0.090	0.817	0,265	0.085
Magnesium	73.8±35.4	85.6±37.6	82.3±37.6	0.114	0.113	0.299	0.864
***Mg/Cr**	**58.0±22.5**	**66.1±23.4**	**69.1±28.6**	**0.012**	0.081	**0.012**	0.736
***K/Cr**	**39.3±15.1**	**42.7±19.3**	**51.3±34.7**	**0.009**	0.587	**0.008**	0.095
pH	6.1±0.9	6.0±0.9	6.1±0.9	0.6698	0.7916	0.9705	0.6663
Volume	1927±614.5	2028.8±790.6	2014.7±656.6	0.4361	0.4044	0.8567	0.7481

SD = standard deviation; **K** = potassium; **Mg** = magnesium; **Cr** = creatinine

Table 2 - Metabolic alterations in 24-hour period urine at baseline and when taking *P. niruri* and Wash out.

Metabolic alteration	Measure Urine 24- hour	Reference	Baseline	MEAN (SD) *P. niruri* mg/ vol.24-hour	Wash out	ANOVA P
Hypercalciuria (n=24)	Calcium	<240/200*	300.3 ± 106.6	237.2 ± 70.7	227.5 ± 79,3	0.2619
Hyperuricosuria (n=6)	**Uric acid**	**<0.75/0.6***	**0.77 ± 0.2**	**0.54 ± 0.1**	**0.56 ± 0.12**	**0.0057**
Hypocitraturia (n=24)	Citrate	>290/320*	211.8 ± 123.7	322.3 ± 147.3	345.3 ± 147.3	**0.2193**
Hyperoxaluria (n=5)	**Oxalate**	**<40**	**59.0 ± 11.7**	**28.8 ± 16.0**	**33.0 ± 4.4**	**0.0002**
Hypernatriuria (n=34)	Sodium	<150	211.6 ± 62.8	183.5 ± 92.0	200.6 ± 59.7	0.1770

***** = value reference for men/women; **SD** =standard deviation

Table 3 - Number and size of upper urinary calculi in patients treated with *P. niruri*.

Measure	MEAN (SD)			ANOVA P	TUKEY		
	Baseline	*P. niruri*	Wash out		Baseline x *P. niruri*	Baseline x Wash out	*P. niruri* x Wash out
Total calculi (n)	3.2±2.0	2.0±2.1	2.2±2.2	**0.0005**	**0.0005**	**0.015**	0.672
Right kidney (n)	1.6±1.4	1.1±1.2	1.2±1.4	**0.0176**	**0.0176**	**0.201**	0.587
Left kidney (n)	1.6±1.4	0.9±1.1	1.0±1.1	**0.0003**	**0.0003**	**0.003**	0.938
Size (mm)	15.6±10.6	9.4±8.9	11.2±11.1	**0.0002**	**<0.0001**	**0.045**	0.206

SD = standard deviation; **N** = 56

high worldwide with an increase in morbidity and health costs in a number of countries. However, fully effective treatment and prevention have yet to be established, and the use of medicinal plants may be helpful as coadjuvants. The use of *P. niruri*, a very common plant found in different countries, has been shown to be a viable alternative, though more clinical studies are necessary.

In the present study, we sought to evaluate the use of *P. niruri* in patients with small urinary stones. When we analysed urinary variables, we found no significant changes in urinary volume for a 24-hour period with the use of an infusion of the plant. The urine volume was close to the minimum recommended in the literature, which is 2L/day (3, 4). The patients in this study exhibited average values of 1927mL, 2029mL, and 2015mL daily for the baseline, *P. niruri* and washout periods, respectively. It is possible that the patients were aware of the importance of fluid intake to prevent the formation of new calculi and were thus already consuming the quantity of liquid recommended at the beginning of the study; this would have resulted in no significant change in urine volume. The diuretic effects of *P. niruri* have been described in experimental studies (10, 15), though Nishiura et al. (11) reported different results in a clinical study.

The increase in the 24-hour urine electrolytes found in this study may be related to the decrease in the number of calculi during the imaging evaluation. Increases in urinary potassium and magnesium levels lead to alkalinisation of the urine and consequently to an increase in urinary citrate, a potent inhibitor of calcium stone formation (5).

Potassium can moderate the concentration of sodium in urine and promote the elevation of citrate, which acts to correct urinary pH and acidity, possibly contributing to an increase in calcium solubility (5). These changes may interfere with some stages of crystallization in urine, such as a reduction in the nucleation, growth and aggregation of calcium oxalate crystals (5, 14). We

observed a significant increase in potassium in 24-hour period urine samples between the baseline and washout periods, which was also noted in the change in the potassium/g Cr 24-hour ratio and in the change in the magnesium/g Cr 24-hour ratio. The increased potassium and magnesium levels observed in the study patients may help to explain the normalization of metabolic changes observed following the use of *P. niruri*.

Urine pH in the sample urinalysis did not change throughout the study and remained at a mean value between 6.0 and 6.1.

Diastolic blood pressure showed a significant reduction between the *P. niruri* and washout periods (16). A report of a hypotensive effect of *P. niruri* can be found in the literature (8).

The calculus imaging evaluation performed showed a reduction in the number of calculi after the *P. niruri* stage, as shown in Table-3, considering that the number of stones shown on ultrasound was lower due to the fact the some patients probably passed some of them during the study.

Although the same method of evaluation was utilized in the study performed by Nishiura et al., those authors did not show a change in the number and size of calculi before and after the use of a *P. niruri* extract in individuals with urinary lithiasis (11). In contrast, it has been reported that *P. niruri* promoted a reduction in the number of calculi and their appearance in relation to the most fragile aspect of the calculi structure (7, 17-19).

Although ultrasonography is not considered a gold standard for the evaluation of small calculi, we performed this evaluation for the purpose of clinical monitoring of the patients because repeated CT scans in a short period of time can result in undue radioactivity exposure, which is not recommended (20).

Some patients experienced haematuria and abdominal pain. The exact cause of these events not was clear and continuous monitoring of patients at monthly visits was implemented as a precaution during the study. The haematuria and abdominal pain may be related to small calculi eliminated during that period, as patients observed the presence of sand fragments in the urine. Regardless, we cannot attribute the pain reported to the consumption of *P. niruri* because this symptom is very frequent in patients with lithiasis.

In this study, there were no changes in serum levels of liver enzymes, urea and serum creatinine. In previous experimental studies, no adverse acute or chronic toxic effects, such as kidney, heart, liver or neurological effects, were reported with the use of *P. niruri* (17, 18). In a human study, Wang et al. (21) found the normalization of liver enzymes when the plant was used in patients with chronic liver disease. However, Nishiura et al. (11) reported no change in serum and urinary parameters when analysing 69 patients and a control group with and without the use of *P. niruri* (11).

Most studies to date have focused on the hepatoprotective effects of *P. niruri*, and the genus Phyllanthus has been utilized in the treatment of various liver disorders (8). The reduction in alkaline phosphatase levels observed in this study was beneficial. In addition to showing that there was no toxicity with the use of the plant extract, this decrease may have contributed to the reduction in the number of calculi at the end of the study, because alkaline phosphatase is a biochemical marker of bone metabolism and may be involved in the mechanism of calculus formation in hypercalciuria (22).

According to our evaluation of patients with metabolic alterations at the baseline and *P. niruri* periods, a significant normalization of urinary uric acid and oxalate values occurred in those with hyperuricosuria and hyperoxaluria, respectively. Citrate levels tended to norma lize in the same cohort, though without statistical significance, likely due to the small sample size. Moreover, no significant change was noted for patients with hypercalciuria or hypernatriuria at the basal stage.

Overall, clinical studies with more patients are needed to validate the use of *P. niruri* in daily practice, particularly in patients with baseline urinary metabolic disorders. *P. niruri* is an abundant natural resource available in many countries, and it can reduce the health system expenses associated with conventional drugs, which are often inaccessible to the majority of the population for long-term treatment.

CONCLUSIONS

P. niruri intake is safe and does not cause significant adverse effects or significant serum

metabolic changes. The use of the tea of this plant increases urinary excretion of magnesium and potassium. Patients with specific urinary metabolic changes such as hyperuricosuria and hyperoxaluria may benefit from ingestion of this tea.

CONFLICT OF INTEREST

None declared.

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ORIGINAL PAPER

Phyllanthus niruri and Chrysanthellum americanum in association with potassium and magnesium citrates are able to prevent symptomatic episode in patients affected by recurrent urinary stones: A prospective study

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Summary

Objective: *The aim of this study is to evaluate the efficacy of a food supplement containing Phyllanthus niruri and Chrysanthellum americanum in association with potassium and magnesium citrates in the treatment and prophylaxis of urinary stones.*

Materials and methods: *Eighty-two patients (mean age 49.7 ± 11.2) with history of urinary stones received this food supplement, one capsule a day for 6 months. Each administration contained a combination of the following ingredients: 244 mg Potassium citrate, 735 mg Magnesium citrate, Phyllanthus (Phyllantus niruri) herb d.e. 15% mg Tannins 220 mg, Chrysanthellum (Chrysanthellum americanum Vatke) plant d.e. ¼ 55 mg. After 6 months, all patients underwent urologic visit, urinalysis, imaging and quality of life (QoL) questionnaires evaluation. Each patient was also evaluated by computed tomography (CT) scan at baseline and at 6 months.*

Result: *From January 2018 to March 2019, 82 patients (mean age 49.7 ± 11.2) completed the follow-up period and were analyzed. Fifty patients showed lower stone dimensions (60.9%). The average stone size was 0.9 mm, with a significant reduction in comparison with the baseline (-6.7 mm ± 3 mm) ($p < 0.001$). Forty-nine patients (59.7%) did not show any symptomatic episode with an improving in QoL (+0.4 ± 0.1) ($p < 0.001$) in comparison with the baseline. At the end of the follow-up period, 27 patients out of 82 were stone-free (32.9%). Moreover, we report a significant reduction of patients with asymptomatic bacteriuria (ABU) between the baseline and the end of the follow-up evaluation ($p < 0.001$).*

Conclusions: *In conclusion, this food supplement is able to improve quality of life in patients with urinary stones, reducing symptomatic episodes and the prevalence of ABU.*

KEY WORDS: *Stones; Phyllanthus niruri; Chrysanthellum americanum; Potassium; Magnesium; Asymptomatic bacteriuria.*

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INTRODUCTION

Urinary tract stones are one of the most common cause of urological visits, with a prevalence among urological patients of 1-15% (1, 2). The impact on everyday clinical practice is high, due to the high number of recurrences and due to the impact of symptoms on patients' quality of life (2, 3).

The recurrence rate of urinary calculi is 50% within 10 years of the first episode (3, 4). Symptomatic recurrence episodes are associated with high direct and indirect costs (admission to emergency departments, imaging, drugs and working day lost). For these reasons, the prevention of symptomatic episodes due to urinary stones should be the first aim in the management of this kind of patients. Several authors recommended some diet interventions for reducing the risk of urinary stone formation and its recurrence but there is no conclusive consensus in the literature regarding the effectiveness of dietary interventions and recommendations about specific diets for patients with urinary calculi (5). On the other hand, the use of medicinal plants and nutraceuticals have long been used worldwide for the management of recurrence in patients affected by urinary stones (6). *Ettinger et al.*, published in 1997 one of the first clinical trial on the use of potassium-magnesium citrate in preventing recurrent calcium oxalate kidney calculi, demonstrating that potassium-magnesium citrate effectively prevents recurrent calcium oxalate stones (7). Focusing on the plant extracts, *Phyllanthus niruri*, commonly known as "*stone-breaker*", is able to increases urinary excretion of magnesium and potassium and to cause a significant decrease in urinary oxalate and uric acid in patients with hyperoxaluria and hyperuricosuria, contributing to the elimination of urinary calculi (4). Moreover, *Chrysanthellum americanum* seems to be effective in the reduction of stone formation, probably due to the effect of chrysantellin, a saponin, on the stone aggregation (8).

Starting from these evidences, we aim to evaluate the efficacy of a medical device containing *Phyllanthus niruri* and *Chrysanthellum americanum* in association with potassium and magnesium citrates in the treatment and prophylaxis of symptomatic episode in patients affected by recurrent uncomplicated urinary stones.

No conflict of interest declared.

Materials and methods

Study schedule and population

From January 2018 to March 2019, all patients attending two referral institutions with history of recurrent uncomplicated urinary stones were enrolled in this prospective phase IV, post-marketing clinical trial. All enrolled patients underwent a urological visit for inclusion and exclusion criteria assessment with *quality of life* (QoL) questionnaires, serum chemistry, urinalysis and non-contrast-enhanced CT scanner. All patients were encouraged to make lifestyle changes and received a food supplement containing *Phyllanthus niruri* and *Chrysanthellum americanum* in association with potassium and magnesium citrates, one capsule a day for 6 months. After 3 months, all patients were contacted by phone by the trialists in order to check the adherence to the treatment. After 6 months, all patients underwent urologic visit, urinalysis, imaging (CT scan) and QoL questionnaires evaluation. Figure 1 shows the study schedule.

Outcome measures

The main outcome measures were the reduction of symptomatic episodes and improvement in questionnaire result from baseline at the end of the follow-up period. Stone dimension reduction and stone-free status at 6 months follow-up CT scan were also considered as secondary outcome measures.

Inclusion and exclusion criteria

We considered for the inclusion, all patients with CT demonstration of one or multiple renal stones up to 15 mm. All patients with the following characteristics were excluded: serum creatinine level > 1.6 mg/dL, microbiological demonstration of urinary tract infection, non-controlled diabetes, chronic liver disease and all the other serious comorbidities. Moreover, all patients with the evidence of ureterohydronephrosis or renal colic were excluded. Pregnant women were excluded, too.

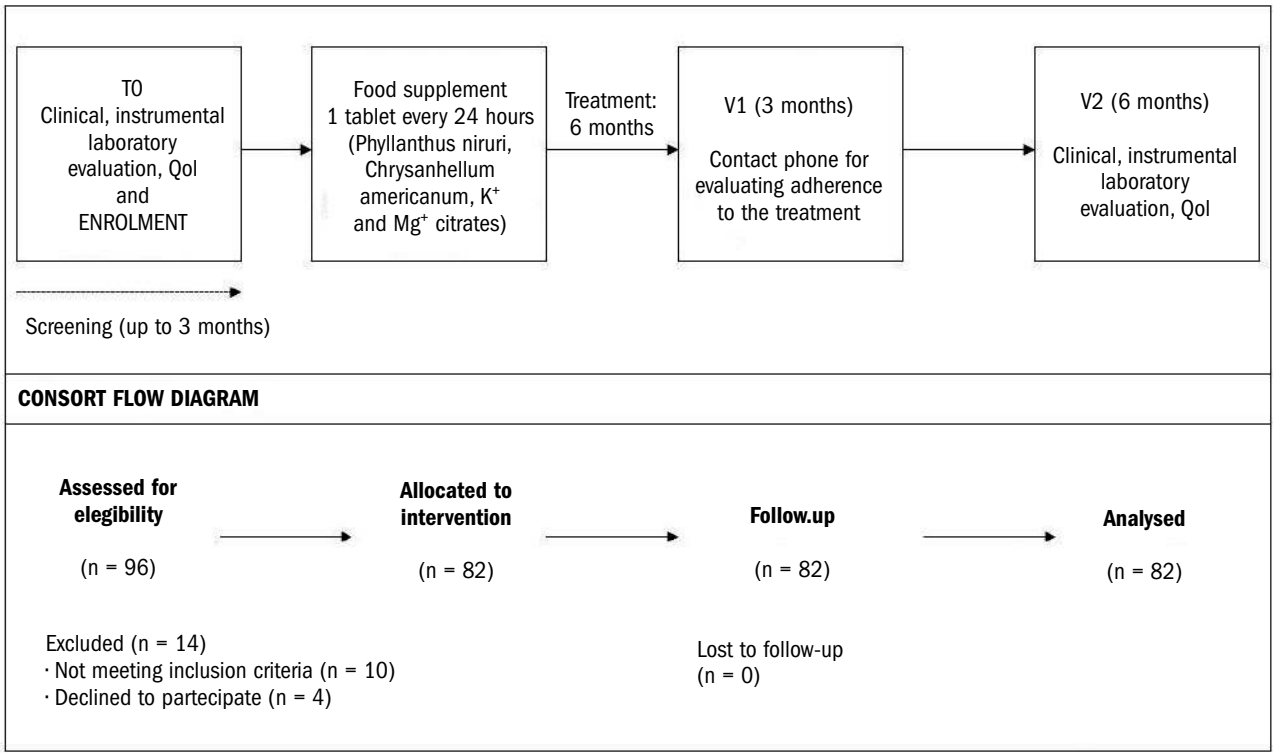
Patients' clinical, laboratory and instrumental assessment

At the baseline, all patients underwent urological visit with QoL questionnaires, serum chemistry and blood analysis, urinalysis and non-contrast-enhanced CT scan. The identified calculi were classified according to their number, location (superior, middle, inferior calyx) and size. Clinical data comprised systolic and diastolic blood pressure and anthropometric evaluation (weight, height, *body mass index* (BMI). Serum chemistry and blood analysis comprised blood count, assessment of urea, creatinine, sodium, potassium, glucose, uric acid, total and ionized calcium, total cholesterol and fractions, triglycerides, alanine aminotransferase, aspartate aminotransferase, gamma-glutamyl transpeptidase, amylase and bilirubin levels. Urinalysis with urinary pH measurement and a urine culture were performed using spontaneous voided urine. Moreover, before enrolment all patients received a screening evaluation which includes a medical and dietary history by an experienced nephrologist.
The enrolment has been done on the basis of the baseline metabolic profile, as suggested by the international guidelines (5).

Questionnaires

The impact of symptomatic episodes of renal stones on patients' QoL has been evaluated by using an Italian version of the Quality of Well-Being, a validated, multi-attribute health scale (9). This scale was selected because

Figure 1.



it has been successfully applied to acute illnesses, whereas other quality of life scales, including the *Short Form-36* (SF-36) Health Survey, are more suitable in chronic cases. Higher scores on the QoL scale reflect a higher quality of life (10).

Follow-up and efficacy assessment

After treatment, all patients were reassessed by urologic visit with questionnaire, serum chemistry, urine analysis and imaging, in order to evaluate the changes in number, location and size of the calculi at 3 and 6 months. At 3 months all patients underwent urinary tract sonography and at 6 months non-contrast-enhanced CT scanner.
At 3 months the stone evaluation was < performed by using urinary tract sonography in order to reduce the patients' exposition to X-ray. However, the stone free-rate has been calculated between the baseline and the 6 months follow-up CT scan.

Compounds characteristics

All patients were treated in line with the manufacturer's instructions (*Erbozeta S.p.A., RSM - https://www.erbozeta.com*). Each administration contained a combination of the following ingredients: 244 mg potassium citrate (93 mg potassium and 150 mg citrate), 735 mg magnesium citrate (30 mg magnesium and 160 mg citrate), *Phyllanthus* (*Phyllantus niruri*) herb d.e. 15% mg *Tannins* 220 mg, *Chrysanthellum* (*Chrysanthellum americanum Vatke*) plant d.e. ¼ 55 mg.

Statistical analysis

As null hypothesis, we consider that there is no difference in terms of number of symptomatic episodes and QoL between baseline and the end of the follow-up evaluation. In order to obtain significant results to analyze, sample size calculation was based on the following assumptions: difference in terms of QoL between baseline and follow-up visit: + 0.3 ± 0.06; α error level, 0.05 two-sided; statistical power, 80%; anticipated effect size, Cohen's d = 0.5. The calculation yielded 72 individuals. Considering a drop-out rate of 10%, the final sample size was set to 79 patients. The statistical analysis was performed as follows: continuous variables are presented as median and *InterQuartile Range* (IQR) and categorical variables are presented as absolute (n) and relative (%) frequency distributions. t-test were used to compare average performance between enrolment and the follow-up evaluation, and between the periods before and after enrolment.
The statistical analysis was performed using SPSS.

Ethical considerations

Due to the fact that this food supplement is already present in Italian pharmacopeia and that Phyllantus niruri has been approved for the management of patients affected by urinary stones in Italy, the study did not require approval by the local ethics committee (IRB). Nevertheless, our study was conducted in line with Good Clinical Practice guidelines and the ethical principles laid down in the latest version of the Declaration of Helsinki. Before inclusion, all participants signed the written informed consent about personal data collection and storage, in accordance with national bylaws.

All anamnestic, clinical and laboratory data containing sensitive information about patients were de-identified in order to ensure analysis of anonymous data only.
The de-identification process was performed by non-medical staff by means of dedicated software. A placebo run-in period was considered unnecessary.

RESULTS

From January 2018 to March 2019, 82 patients (mean age 49.7 ± 11.2) completed the follow-up period and were analyzed.

Baseline

At the baseline, the average stone size was 7.8 mm and 23 patients showed (ABU). The most common isolated strain was *Enterococcus faecalis* (18/23; 78.2%).
The median number of symptomatic episodes at baseline was 3 per year. The median number of calculi was 1 (range: 1-4). In 38 patients (46.3%) the calculi were located in the right kidney, 36 (43.9%) had left kidney lithiasis, while 8 patients had bilateral kidney lithiasis (9.8%). The mean stone diameter was 7.8 ± 1.1 mm.
The mean Hounsfield units was 637.1 ± 264.8, in all enrolled patients. No difference, from the normal values, has been reported in terms of urine analysis parameters among the enrolled patients. The Table 1 shows all demographic and baseline clinical and instrumental characteristics of enrolled patients.

Table 1.
Patient clinical, instrumental and laboratory characteristics at the baseline.

No. of enrolled patients	82
Median age (± SD)	49.7 ± 11.2
Sex	
Male	49 (59.7)
Female	33 (40.3)
Body Mass Index (BMI) (± SD)	25.8 ± 6.3
Charlson Comorbidities Index	
0	75 (11.5)
1	7 (8.5)
2	-
Start of urinary lithiasis (years) (range)	2.3 ± 0.9
Number of symptomatic episodes per year (± SD)	3 ± 1.2
History of any endourological treatment (in the last 6 months)	
Yes	12 (14.6)
No	70 (85.4)
Median number of stones (range)	1 (1-4)
Mean stone size (± SD) (mm)	7.8 ± 1.1
Stones side	
Right kidney	38 (46.3)
Left kidney	36 (43.9)
Bilateral	8 (9.8)
Mean Hounsfield Units (± SD)	637.1 ± 264.8
Stones location (calyx)	
Superior	30 (36.5)
Middle	38 (46.4)
Inferior	14 (17.1)
Presence of asymptomatic bacteriuria	
Yes	23 (28.1)
No	59 (71.9)
Isolated strains	
Enterococcus faecalis	18 (78.1)
Escherichia coli	3 (13.3)
Klebsiella spp.	2 (8.6)

6 months follow-up

Adherence to the life-style changes and treatment

At the 3 months follow up by telephone call, 50 patients reported a high adherence to the life-style changes and to the treatment. At the end of the follow-up period, the adherence to the life-style changes and to the treatment was total in 60 patients (73.1%), while in twenty-two patients (26.8%) some minimal missing doses have been registered. A significant improvement has been reported between the 3- and 6-months evaluations in terms of adherence to the treatment and the life-style changes.

Clinical and instrumental outcomes and QoL

Twenty-seven patients out of 82 had no evidence of stone at the non-contrast-enhanced CT scanner (32.9%) at the end of the follow-up evaluation, reporting stone expulsion, while 50 patients showed lower stones dimension (60.9%).

The average stones size at the end of the follow-up was 0.9 mm (± 0.1 mm), with a significant reduction in comparison with the baseline (-6.7 mm ± 3 mm) ($p < 0.001$). Forty-nine patients (59.7%) did not show any symptomatic episode with an improving in QoL (+0.4 ± 0.1) ($p < 0.001$) in comparison to the baseline. No difference, from the normal values, has been reported in terms of urine analysis parameters among the enrolled patients. The Table 2 shows all clinical and instrumental findings at the end of the follow-up.

Asymptomatic bacteriuria

At the end of follow-up evaluation, 3 patients reported ABU. A significant reduction of patients with ABU between the baseline and the end of the follow-up evaluation (23 *vs* 3; $p < 0.001$) has been reported.

A significant correlation between ABU reduction and stones number and dimension reduction has been reported ($r = 0.83$; $p < 0.001$).

Adverse effects

No mild or severe clinically significant adverse effects have been reported.

Table 2.

Clinical, instrumental and laboratory findings at each follow-up visit (3 and 6 months after treatment).

	Baseline	3 months	6 months
Adherence to life-style changes recommendations			
Patients with a high Grade of adherence	-	50 (60.9)	60 (73.1)
Clinical improvement			
Recurrence-free patients	0 (0%)	38 (46.3)	49 (59.7)
QoL	97.1	97.7	97.9
QoL improvement	-	+0.3 ± 0.2	+0.4 ± 0.1
Difference from baseline		p < 0.001	p < 0.001
Stone-free status	0 (0%)	19 (23.1)	23 (32.9)
Stone size (mm)			
Stone size (mm)	7.8 ± 1.1	3.1 ± 0.5	1.1 ± 0.5
Stone size reduction		-4.2 ± 2	-6.7 ± 3
Difference from baseline		p < 0.001	p < 0.001
Asymptomatic bacteriuria			
Patients with asymptomatic Bacteriuria	23	9 (10.9)	3 (3.6)
Difference from baseline		p < 0.001	p < 0.001

Discussion

We demonstrated that a food supplement containing *Phyllanthus niruri* and *Chrysanthellum americanum* in association with potassium and magnesium citrates is able to improve QoL in patients with urinary stones, reducing symptomatic episodes, stones number and dimension and the prevalence of asymptomatic bacteriuria. Two important points should be discussed: The role of phytotherapy in the management of urinary stones and its mechanism of action and the relationship between urinary stones and ABU.

Role of phytotherapy in the management of urinary stones and mechanism of action

Phytotherapy is one of the first choices regarding pharmaceutical treatment of patients affected by urinary stones (2) due to the demonstrated efficacy (4, 11) and due to the patient's preference for phytotherapeutic compounds. *Micali and Pucci*, in two clinical trials, demonstrated that *Phyllanthus niruri* is able to reduce the stone size and improve the stone free status rate (4, 11). In line with these trials, we found a significant reduction in terms of stones size in comparison with the baseline (-6.7 mm ± 3 mm) ($p < 0.001$) and a high percentage of stone free patients after treatment.

These interesting findings are probably due to *Phyllanthus niruri* and *Chrysanthellum americanum* combination in association with potassium and magnesium citrates.

The efficacy of *Phyllanthus niruri* is probably augmented by the presence of *Chrysanthellum americanum*.

The role of *Chrysanthellum americanum* in the management of urinary stones is due to its interference, through the chrysantellin, a saponin, with some stages of crystallization in urine, such as a reduction in the nucleation, growth and aggregation of calcium oxalate crystals (8). Moreover, the synergistic effect of *Phyllanthus niruri* and *Chrysanthellum americanum* is probably due to its diuretic effects (12, 13). Furthermore, this food supplement contains potassium and magnesium citrates, although previous studies did not favor potassium citrate therapy. However, potassium can moderate the concentration of sodium in urine and promote the elevation of citrate, which acts to correct urinary pH and acidity, possibly contributing to an increase in calcium solubility and, then, interfere with some stages of crystallization in urine (14-17). This food supplement is, then, able to act into two different pathways: diuresis increasing and inhibition of nucleation, growth and aggregation of calcium oxalate crystals. Moving to the role of life-style changes, we found a significant improvement between the 3- and 6-months evaluations in terms of adherence to the treatment and life-style changes. The clinical reported efficacy in terms of reduction of symptomatic episodes, due to the treatment, drives the adherence to the life-style changes. In this sense, this food supplement should be considered, also, as an interesting tool for driving the adherence to the life-style changes and for obtaining a long-term efficacy on the stone recurrence. The high adherence to the treatment is due to the absence of reported adverse effects, too.

Relationship between urinary stones and asymptomatic bacteriuria

This was the first study that analyzed the efficacy of a

food supplement in reducing ABU in patients affected by urinary stones. ABU is a common clinical condition among recurrent stones patients, which does not generally require any treatment. However, the role of ABU in patients with recurrent urinary stones is not completely understood. Here, we demonstrated that this food supplement is able to statistically significantly reduce the prevalence of ABU among recurrent stone patients.
The efficacy on ABU is probably due to the increase of total daily diuresis and to the inhibition of nucleation, growth and aggregation of bacterial biofilm on the surface of calcium oxalate crystals. This mechanism is probably due to the *Phyllanthus niruri* and *Chrysanthellum americanum* action.
Finally, the role of bacteria biofilm on the urinary stone aggregation is another important field to deeply explore. Future studies are, however, needed to confirm these hypotheses.

Conclusions

In conclusion, this food supplement containing *Phyllanthus niruri* and *Chrysanthellum americanum* in association with potassium and magnesium citrates is able to reduce symptomatic episodes, improving QoL in patients with urinary stones and reduce the prevalence of ABU.

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Can Phyllanthus niruri Affect the Efficacy of Extracorporeal Shock Wave Lithotripsy for Renal Stones? A Randomized, Prospective, Long-Term Study

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Purpose: Phyllanthus niruri is a plant used in Brazilian folk medicine for the treatment of urolithiasis. We assessed the efficacy of P. niruri after extracorporeal shock wave lithotripsy for renal stones.

Materials and Methods: We prospectively evaluated 150 patients with renal stones that were as large as 25 mm and composed of calcium oxalate. All patients received 1 to 3 extracorporeal shock wave lithotripsy sessions by Dornier Lithotriptor S. After treatment 78 of 150 patients (52%) underwent therapy with Uriston®, a P. niruri extract (2 gm daily) for at least 3 months (group 1). Otherwise 72 of 150 patients (48%) were used as a control group (group 2). No significant difference in stone size between the 2 groups was found. Stone clearance was assessed after 30, 60, 90 and 180 days by abdominal x-ray and ultrasound scan.

Results: Stone-free rate (stone-free defined as the absence of any stone or residual fragments less than 3 mm) was 93.5% in group 1 and 83.3% in group 2 ($p = 0.48$) at the end point of the followup (180 days). For lower caliceal stones (56 patients) the stone-free rate was 93.7% in the treatment group and 70.8% in the control group ($p = 0.01$). Re-treatment need for group 1 was 39.7% and for group 2 it was 43.3% ($p = 0.2$). No side effects were recorded with extracorporeal shock wave lithotripsy or P. niruri therapy.

Conclusions: Regular self-administration of P. niruri after extracorporeal shock wave lithotripsy for renal stones results in an increased stone-free rate that appears statistically significant for lower caliceal location. Its efficacy and the absolute lack of side effects make this therapy suitable to improve overall outcomes after extracorporeal shock wave lithotripsy for lower pole stones.

Key Words: kidney calculi, lithotripsy

Extracorporeal shock wave lithotripsy represents a minimally invasive treatment for renal stones. The efficacy and safety of this procedure are well proven and demonstrated. Stone-free rates after ESWL® may vary according to stone size,[1] location[1–3] and the type of lithotriptor used.[4,5] Medical therapies may be used to avoid stone relapse, and these consist traditionally of citrate, potassium and magnesium, which act to correct the imbalance between the promoter and inhibitors of lithogenesis. Other therapies are used to dissolve stones. For many years litholytic agents such as Herniaria hirsuta, kampou extracts and other plants were a common way to treat urinary stones, especially in semi-arid zones and in countries with unreliable water sources.[6–8]

Phyllanthus niruri is a plant traditionally used in Brazilian folk medicine for the treatment of several pathological conditions, including urolithiasis. The plant may act as an inhibitor of crystal growth, because of a higher incorporation of glycosaminoglycans into the stone.[9] Uriston® consists of an extract from P. niruri labeled with specifics tannins. We assessed the efficacy of chronic therapy with Uriston® after ESWL® for renal stones composed of calcium oxalate.

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MATERIALS AND METHODS

From January 2003 to February 2005 we prospectively evaluated 150 patients with renal stones thought to be composed of calcium oxalate. Subjects with urinary tract infections, pregnant women or patients who already underwent renal surgery were excluded from the study. Stones were investigated through abdominal x-ray, ultrasound and excretory urogram. Patients were submitted to 1 to 3 session of ESWL® with the use of Dornier Lithotriptor S.

After ESWL® patients were randomly divided in 2 groups. Group 1 consisted of 78 patients who received an adjunctive treatment with oral medical therapy, and group 2 consisted of 72 patients who did not receive any therapy and served as a control group. The medical therapy of group 1 consisted of 2 gm per day of Uriston®, an extract from P. niruri self-administered once a day for a minimum of 90 days. Both groups were allowed to use symptomatic therapy with ketoprofen on demand and were asked to drink a minimum of 2 liters of water daily. Stone clearance was assessed after 30, 60, 90 and 180 days by abdominal x-ray and ultrasound scan. A stone-free condition was defined as the complete absence of any stone or the presence of residual fragments smaller than 3 mm.

Sampled data were inserted in a database and statistically analyzed with the help of SPSS® 8.0 version for Windows. A complete descriptive analysis of all available vari-

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TABLE 1. *Patient and stone characteristics*

	Group 1	Group 2	p Value
Mean pt age (range)	50 (25–78)	48 (24–73)	
Mean mm stone size (range)	12.2 (5–25)	11.5 (5–20)	
No. stone location:			
Upper caliceal	9	14	0.14
Middle caliceal	16	10	0.22
Lower caliceal	32	24	0.10
Pelvis	21	24	0.68

ables was performed. Statistical analysis was performed with student's t test, the Mann-Whitney U test, the chi-square test and 2-tailed Fisher's exact test. A significance level of 0.05 was chosen for all the tests.

RESULTS

All patients completed the study. Group 1 consisted of 42 men and 36 women (average age 50 years, range 25 to 78), and group 2 consisted of 38 men and 34 women (average age 48 years, range 24 to 73). The average stone size was 12.2 mm (range 5 to 25) for group 1 and 11.5 mm (range 5 to 20) for group 2, with no statistically significant difference between the 2 groups. Stone location in the kidney is reported in table 1. A Double-J® stent was placed before ESWL® in each case in which the stone size was larger than 20 mm. The average number of shock waves per session was 3,000 for both groups (range 2,400 to 3,500). Ultrasound and x-ray were used for focusing on the stone.

Stone-free condition with residual fragments less than 3 mm occurred in 73 (93.5%) of 78 patients in group 1 and in 60 (83.3%) of 72 patients in group 2 at the end point ($p = 0.48$). Stone-free condition without residual fragments occurred in 69 (88.5%) of 78 patients in group 1 and in 55 (76.4%) of 72 patients in group 2 ($p = 0.08$).

Table 2 presents the stone-free rates for different stone locations. Outcomes stratified by stone size and by different steps of followup (30, 60, 90 and 180 days after ESWL®) are reported in table 3. Regarding the stone-free patients, 29 of 73 in group 1 (39.7%) and 26 of 60 in group 2 (43.3%) underwent ESWL® re-treatment ($p = 0.2$). All the collected stones turned out to have a predominantly calcium oxalate composition (greater than 70%).

Medical therapy with Uriston® for group 1 lasted from 95 to 200 days (mean 134). No side effects were noticed during the treatment, neither with ESWL® nor with P. niruri intake. Patients who were not stone-free after 180 days (5 in group 1 and 12 in group 2) were successfully treated with percutaneous lithotripsy.

TABLE 2. *Outcomes stratified by stone location*

	% Group 1 Stone-Free	% Group 2 Stone-Free	p Value
With residual fragments less than 3 mm:			
Overall	93.5	83.3	0.10
Upper caliceal	100	92.8	0.06
Middle caliceal	93.7	90	0.54
Lower caliceal	93.7	70.8	<0.001
Pelvis	90.4	87.5	0.75
Without residual fragments:			
Overall	88.5	76.4	0.08
Upper caliceal	88.8	85.7	0.63
Middle caliceal	87.5	80.0	0.86
Lower caliceal	90.6	62.5	0.03
Pelvis	85.7	83.3	0.85

TABLE 3. *Outcomes stratified by stone size and followup*

	% Group 1 Stone-Free	% Group 2 Stone-Free	p Value
Stone size (mm):			
Less than 10	97.2	84.6	0.02
Greater than 11	90.4	82.6	0.24
Days followup:			
30	54	18.8	0.02
60	69.3	35.2	0.03
90	74.3	64.2	0.38
180	93.5	83.3	0.48

DISCUSSION

ESWL® represents a minimally invasive treatment for renal stones, and its efficacy and safety have improved accordingly with technological progress.[4,5,10] Nevertheless, outcomes of ESWL® may be affected by a number of features connected to both stone and patient characteristics. It is well known that stone size,[1] location[1–3] and composition[11] influence the clearance of residual fragments while a high body mass index compromises the results of stone focusing.[12]

As the technology has improved, some authors have reported outcomes of ESWL® with regard to the type of lithotriptor used. Matin et al found a comparable efficiency quotient between electrohydraulic and electromagnetic units.[4] Sheir et al, with a prospective, randomized comparative study, recently reported an overall stone-free rate of 88.5% and 82.4% for Dornier Lithotriptor S and Dornier MFL 5000, respectively.[5] Other studies investigated ESWL® results based on the location of the stones. Obek et al described an overall stone-free rate of 66% with a Lithostar® lithotriptor with 63%, 73% and 71% stone clearance for lower, middle and upper isolated caliceal stones, respectively.[13]

Lower pole stones still represent a difficult problem due to the reduced discharge of the fragments and their consequent recrystallization. The cause may be based on morphological features of the kidney. Elbahnasy et al described the 3 major radiographic characteristics of the lower pole that influence ESWL® outcomes as infundibulopelvic angle, infundibular length and width.[3] Other clinical studies of 10 to 20 mm lower caliceal stones found stone-free rates varying from 37% to 79.2%.[14,15]

Supportive medical therapy to improve ESWL® results has been attempted in the past by using potassium or magnesium citrates. Cicerello et al suggested that citrate reduces growth or agglomeration, increasing the clearance rate of calcium oxalate.[16] Soygur et al investigated the effect of potassium citrate therapy on the stone recurrence rate and the percentage of residual fragments after ESWL®. In the residual fragment group treated patients had a significantly greater remission rate compared to untreated patients (44.5 vs 12.5%), and no recurrences were noticed at 12 months in the stone-free group following potassium citrate therapy.[17]

In this study we evaluated the efficacy of Uriston®, an extract from P. niruri, after shock wave lithotripsy for renal stones. P. niruri is a plant belonging to the Euphorbiaceae family with a worldwide distribution. It is traditionally used in Brazilian folk medicine in the treatment of urolithiasis. Several authors have investigated its mechanism of action. In

1998 Campos and Schor described the powerful inhibitory effect of P. niruri on CaOx crystal adhesion and suggested that P. niruri intake may reduce urinary calcium in patients with hypercalciuria.[18,19] Barros et al confirmed the inhibition on CaOx crystal growth and aggregation with an in vitro aqueous extract from P. niruri.[20]

Freitas et al suggested that this effect was related to a higher incorporation of glycosaminoglycans into the stone, independently from urinary excretion of citrate and magnesium.[9] In fact, the calculi from a P. niruri treated group had a higher content of GAGs, suggesting that P. niruri reduces the deposition of crystalline particles.

The actual role of GAGs in urolithiasis has been widely evaluated and discussed. GAGs and especially chondroitin sulfate bind to the growth sites of crystals and therefore stop their aggregation. The outcomes of our experience may support all these premises in that residual fragments due to ESWL® seem to have a lower aggregation and crystallization potential.

Complete stone clearance occurred in 93.5% of patients in the treated group but only in 83.3% of patients in the control group. Taking into consideration lower caliceal stones, 93.7% of Uriston® patients were stone-free at 180 days compared to 70.8% of patients in the nontreated group. Stone size ($p = 0.02$) and medical therapy ($p = 0.03$) were the main predictors of stone clearance.

P. niruri reduces the relative risk of ESWL® failure 10.3% overall and 33% for stones located in the inferior calix. Further studies are required to fully understand the role of P. niruri. Nevertheless, clinical outcomes with the use of P. niruri after ESWL® are remarkable as they show a statistically significant increase in ESWL® effectiveness.

CONCLUSIONS

Regular intake of P. niruri after ESWL® results in an increased overall stone-free rate, in a lower re-treatment percentage and in a faster stone clearance. Its efficacy is only evident for stones in the lower caliceal location, where it may act to prevent recrystallization of stone fragments and thus increase their clearance. The effectiveness of P. niruri together with the absolute lack of side effects make this therapy suitable for improving overall outcomes of shock wave lithotripsy for lower pole renal stones.

Abbreviations and Acronyms

CaOx	=	calcium oxalate
ESWL®	=	extracorporeal shock wave lithotripsy
GAG	=	glycosaminoglycans

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Phyllanthus niruri (stone breaker) herbal therapy for kidney stones; a systematic review and meta-analysis of clinical efficacy, and Google Trends analysis of public interest

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Introduction: *Phyllanthus niruri (P. niruri) is the most commonly listed active ingredient in commercially available herbal therapies for kidney stones, despite limited supporting clinical evidence. We performed a meta-analysis to evaluate its efficacy in reducing stone burden. We used Google Trends to analyze its relative popularity in internet searches relative to conventional stone therapies.*

Materials and methods: *A comprehensive literature search for controlled human studies containing data on the effect of P. niruri treatment on stone size and number was performed. Pooled analysis of change in mean stone size and number with P. niruri was performed using a fixed-effects model. Standardized mean difference (SMD) and 95% CI were reported. Google searches in the United States within the "Health" category, for topics "Gale of the wind (P. niruri)", "Extracorporeal shockwave lithotripsy" (ESWL), "Ureteroscopy" (URS), "Laser lithotripsy" (URSL) and "Percutaneous nephrolithotomy" (PCNL), conducted between January 2014 and December 2018, were quantified. Annual median relative search volumes (RSV; 0-100 scale) were compared using the Kruskal-Wallis test. Post-hoc pairwise comparisons were performed using the Dunn test with Holm-Sidak adjustment.*

Results: *Two studies met inclusion criteria. P. niruri treatment resulted in significant decreases in mean stone size (SMD -0.39 cm, 95% CI = -0.68 to -0.09, p = 0.01) and number (SMD -0.38, 95% CI = -0.68 to -0.09, p = 0.01). Median RSV for P. niruri was similar to that for ESWL, PCNL and URS through 2015, but was significantly higher than for ESWL and PCNL after 2015, and higher than for URS after 2016 (each p value p ≤ 0.0012).*

Conclusions: *Limited clinical evidence supports modest efficacy of P. niruri in reducing stone burden, pending further study. Public interest in P. niruri is growing within the United States, possibly reflecting a rising demand.*

Key Words: urolithiasis, naturopathy, internet

Introduction

Kidney stones currently afflict nearly 1 in 11 adults in the United States, often imparting significant patient morbidity, and resulting in detriment to health-related quality of life and high treatment-related costs.[1-3] Recurrence rates exceed 50% within 10 years of the index stone,[4] leading many sufferers to seek preventive management, including dietary and pharmacologic therapy. Additionally, many urolithiasis patients seek out medical chemo-dissolution therapy so as to avoid surgery, but medications for this purpose are limited to date. Currently, the only chemolytic therapies endorsed by the American Urological Association (AUA) are urinary alkalinization for uric acid and cystine stones, cystine-binding drugs for cystine stones, and in rare cases, urease inhibitors for strutvite stones, but no agents for chemolysis of calcium stones are currently recommended.[5] Accordingly, many chronic stone formers seek out non-conventional alternatives, including herbal remedies. Several herbs used in Ayurvedic, Chinese

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and European herbal medicine have been described for this purpose,[6-12] many with purported claims of ability to dissolve stones despite lack of clinical evidence. *Phyllanthus niruri* (*P. niruri*) is a widely available herbal remedy for kidney stones, commonly used in Brazil,[13] and is also widely commercially available in the United States as "Stone Breaker". It is also a key ingredient in numerous other commercially available herbs such as "Chanca Piedra", "Stone Crusher" and "Disolvatol" to name a few. It has been widely evaluated in *in vitro* and *in vivo* pre-clinical studies, as well as a handful of clinical trials.[14] However, data on its clinical efficacy with regard to stone elimination or reduction in size and number are conflicting.[13,15] Additionally, quantitative data on the public's demand for *P. niruri* are lacking. We performed a systematic review and meta-analysis of the available clinical literature on *P. niruri*, with the primary goal of evaluating its efficacy in reducing stone burden (size and number). Additionally, using Google Trends methodology, we quantitated the public's interest in *P. niruri*, by analyzing internet search volume for it relative to search volume for conventional kidney stone surgeries.

Materials and methods

Systematic review and meta-analysis

The systematic review was performed following the Preferred Reporting Items for Systematic Reviews and Meta-Analysis (PRISMA) checklist whenever possible.[16] PubMed, Google Scholar, the Cochrane Library, and SCOPUS databases were searched, and articles reporting on effects of *P. niruri* on kidney stones, published through March 31, 2019 were selected for further screening. We included controlled studies performed in humans, reporting data on the effect of *P. niruri* treatment on stone size and number, published in English. We excluded pre-clinical *in vitro* and *in vivo* studies, review articles, toxicity studies and studies reporting on effects of *P. niruri* other than for kidney stone treatment. The literature search was independently performed by 2 reviewers, with data extracted into identical extraction tables. Pooled analysis of change in mean stone size and number following *P. niruri* treatment was performed using a fixed-effects model, with standardized mean difference (SMD) and 95% confidence interval (CI) reported. Heterogeneity was assessed by calculating the I^2 value.

Google Trends methodology

Google Trends is a free, publically accessible online portal that enables quantitation of the volume of internet searches for a user-specified topic. It is increasingly used in health care to gain insights into disease epidemiology and population health behaviors.[17,18] Search words can be defined as "terms" or "topics"; "term" searches return matches on all terms entered in the language given, while "topics" searches group together several terms that share a common concept, in any language. Absolute number of internet searches on a specified subject is divided by the total volume of searches on all topics conducted during the same specified time period within a specified geographic region. Relative search volume (RSV) for each search subject, a measure of its relative popularity, is reported on a 0-100 scale.[19]

We determined RSV for the following topics: "Gale of the wind" (*P. niruri*), "Extracorporeal shockwave lithotripsy" (ESWL), "Ureteroscopy" (URS), "Laser lithotripsy" (URSL) and "Percutaneous nephrolithotomy" (PCNL), for searches performed in the United States between January 1, 2014 and December 31, 2018, under the query category "Health". Search data were downloaded on February 3, 2019. Median RSV values in each year were compared across the above 'topic' categories using the Kruskal-Wallis test. Post-hoc pairwise comparisons of RSVs for the treatment modalities were performed using the Dunn test with Holm-Sidak adjustment for multiple comparisons. Stata version 15 (StataCorp LP, College Station, TX, USA) statistical software was used for statistical analyses.

Results

Study selection

A total of 21 articles of interest were retrieved from the database searches. Of these, 10 were review articles, 4 were *in vivo* animal studies and 4 were *in vitro* studies, resulting in exclusion. Only 3 clinical studies were identified, including 2 randomized controlled trials (RCTs)[13,20] and one prospective controlled study.[15] Of the RCTs, one reported on the effect of *P. niruri* on stone clearance rates following ESWL, but did not contain data on stone size and number[20] and was therefore excluded from the final analysis. Only two studies, one with 56 patients[15] and the other with 69 patients,[13] contained data on changes in stone size and number following treatment with *P. niruri*, in both cases as secondary endpoints, Figure 1. The pooled data from these studies included 89 patients treated with *P. niruri* versus 92 controls who received placebo or no treatment.

Effect on stone size and number

In study by Pucci et al 56 patients with radiographically confirmed stones, underwent 12 weeks of treatment with *P. niruri* followed by a 12-week washout. Each

Figure 1. Study flow diagram.

patient served as their own control. Twenty-four hour urines and renal ultrasounds were obtained at baseline, immediately after *P. niruri*, and at the end of the washout phase,[15] and kidney stone size and number were documented for each phase. Compared with baseline, treatment with *P. niruri* resulted in significant reduction in mean (SD) number of stones from 3.2 ± 2.0 to 2.0 ± 2.1 (p = 0.0005), as well in reduction in mean stone size from 1.56 ± 1.06 cm to 0.94 ± 0.89 cm

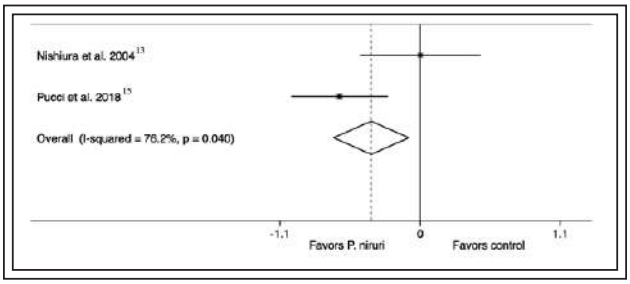

Figure 2. Meta-analysis of *P. niruri* effect on size of stones.

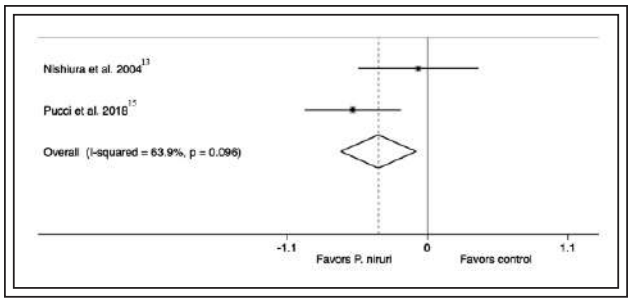

Figure 3. Meta-analysis of *P. niruri* effect on number of stones.

(p = 0.0002). These effects were maintained during the washout phase. In study by Nishiura et al, 69 calcium stone formers were randomized to receive *P. niruri* (n = 33) versus placebo (n = 36) for a period of 3 months. Serum and 24 hr urine chemistries, as well renal ultrasonography were obtained at baseline and at the end of the study. In the *P. niruri* group, mean (SD) stone size and number post-treatment (0.6 ± 0.2 cm and 1.5 ± 1.4 respectively) did not significantly differ from pre-treatment values (0.6 ± 0.2 cm and 1.8 ± 0.9 respectively) or from placebo.[13]

In the pooled analysis, data for a total of 89 patients exposed *P. niruri* versus 92 controls were compared. Treatment with *P. niruri* was found to result in a significant decrease in mean stone size compared with placebo or no treatment (SMD -0.39 cm, 95% CI = -0.68 to -0.09, p = 0.01; I^2=76%), Figure 2. *P. niruri* treatment also resulted in a significant decrease in number of stones compared with placebo or no treatment (SMD -0.38, 95% CI = -0.68 to -0.09, p = 0.01; I^2=64%), Figure 3.

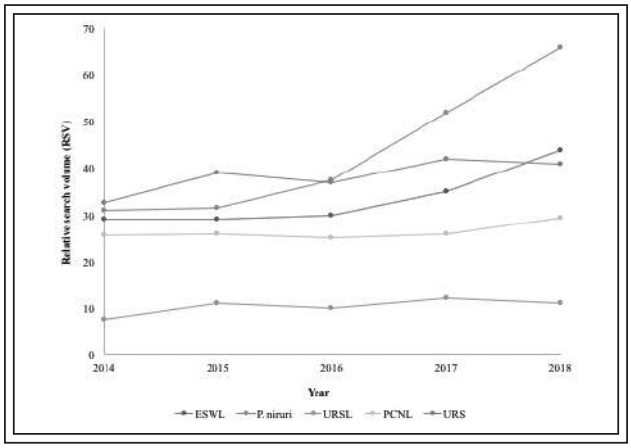

Figure 4. Internet search volume for *P. niruri* in comparison with conventional surgical therapies for kidney stones.

Google Trends data

Median RSV for *P. niruri*, ESWL, URS and PCNL was significantly higher than that for URSL in each year between 2014 and 2018 ($p < 0.0001$ in each case). For 2014 and 2015, median RSV for *P. niruri* was similar to that for ESWL and PCNL ($p > 0.05$ in each case), but for 2016 to 2018, median RSV for *P. niruri* significantly exceeded that for ESWL and PCNL in each year ($p \leq 0.0004$ in each case). Median RSV for *P. niruri* was similar to that for URS during 2014-2016 ($p > 0.05$), but significantly higher than for URS in 2017 and 2018 ($p \leq 0.0012$ in each case), Figure 4.

Discussion

Data from a 2007 National Health Interview Survey (NHIS), conducted by the National Center for Complementary and Integrative Health (NCCIH), a branch of the National Institutes of Health (NIH) reveal that approximately 38% of adults in the United States use some form of complementary and alternative medicine for a wide range of health conditions . Estimated expenditures for these therapies among United States adults totaled $13 billion in 1993, majority paid out-of-pocket.[21] *P. niruri* is the main ingredient of several naturopathic formulations for kidney stone treatment in commercial use in the United States. It is native to tropical areas and has been widely used worldwide for more than 2000 years in treating kidney stones.[22] In a recent review, Boim et al outlined much of the pre-clinical evidence supporting the possible beneficial role of *P. niruri* in kidney stone treatment.[14] *In vitro*, it was found to decrease calcium oxalate (CaOx) crystal size and aggregation, inhibit crystal adhesion and endocytosis, and to inhibit xanthine oxidase activity. *In vivo*, it was found to reduce the rate of CaOx stone growth independently of modifying other lithogenic factors, and to disrupt crystal-matrix interactions, resulting in smoothing of the surface of already formed CaOx stones.[14]

Anecdotally, many stone formers who use "stone breaker" believe that it is effective in dissolving kidney stones. In the present study, we analyzed the available clinical evidence for the efficacy of *P. niruri* in reducing stone burden. We found that although clinical studies are lacking overall, two prospective controlled studies, including one RCT, analyzed the effects of *P. niruri* on stone size and number as a secondary outcome.[13,15] In the pooled analysis of the data from these trials, *P. niruri* treatment over at least 3 months resulted in modest, statistically significant reductions in the size and number of calcium stones. However, the pooled comparisons were characterized by significant heterogeneity ($I^2 = 76\%$ for stone size and 64% for stone number), which we believe is partly due to the fact that the outcomes evaluated in our analysis were not the primary outcomes assessed in the individual studies, and the selection of control groups varied between the trials. Nonetheless we believe that the use of a fixed effects meta-analysis was justified, in that the two trials both sought to measure near-identical primary and secondary endpoints, each used *P. niruri* in the intervention arms for a period of 3 months, and each obtained *P. niruri* using near-identical extraction methods from the natural plant i.e. tea prepared from the dry plant extract, which was delivered using different modalities (tea capsule versus infusion tea). In a separate RCT comparing stone clearance rates post-ESWL for those receiving adjuvant *P. niruri* versus no additional therapy, Micali et al observed that although there was no overall difference between the groups in the overall stone free rate, stone free rates for patients with lower pole stones were significantly higher for those who received *P. niruri* therapy.[20] Collectively the above findings suggest that *P. niruri* may have a clinically significant role in reducing stone burden, but additional larger clinical studies are needed to better explore its efficacy.

Our analysis of search data available through Google Trends revealed that searches for *P. niruri* were as popular, and in more recent years significantly more popular than searches for conventional kidney stone treatments performed during the corresponding time period in the United States. Google Trends is a relatively novel tool which is increasingly used in epidemiological research to gain insights into health information seeking behavior of populations, methodology sometimes referred to as "infodemiology".[18,23] Use of internet-based data is recognized by the Institute of Medicine as an emerging tool that has the potential to complement and extend more traditional epidemiologic surveillance methods.[24] In the setting of nephrolithiasis, Google search data have been found to strongly correlate with data for kidney stone hospitalizations available through the National (Nationwide) Inpatient Sample (NIS) database.[25] Our findings point to growing information seeking behavior within the United States public for *P. niruri* in recent years. Considering that subjects who seek information about a personal health problem are 60% more likely to contact a health professional following search,[26] we postulate that for a large proportion of people, seeking information about *P. niruri* will likely translate towards future efforts to acquire it for therapeutic purposes, suggesting a growing public demand despite limited supporting clinical evidence.

Although to our knowledge our study is the first to evaluate the clinical efficacy of *P. niruri* by pooled analysis of the available evidence, and also the first to examine

the public's information seeking behavior for this herb relative to conventional treatments for stone disease, it is not without its limitations. Firstly, the number of clinical studies and treated patients is small, limiting the power to estimate its true clinical impact. Nonetheless we believe that the totality of the evidence from completed studies to date points to a potential benefit, and that further larger-scale studies are warranted. Secondly, search results for *P. niruri* on the Google Trends platform could have included searches for its use in health conditions other than kidney stones, resulting in overestimation. However, *P. niruri* is most commonly used worldwide for kidney stone treatment, having been used for this purpose for over 2000 years,[22] and we therefore believe that a majority of the searches were with regard to its use for kidney stones. Third, although insights into the public's demand for *P. niruri* were obtained by analysis of internet search data, its actual utilization as a treatment alternative in stone disease was not directly measurable. Nonetheless taking into consideration the observations regarding trends in internet search behaviors, the widespread, centuries-long use of *P. niruri* for kidney stone treatment, and anecdotal evidence, we believe there is a potentially high and growing demand for it, which could likely continue to rise.

Conclusion

Based on limited clinical evidence, *Phyllanthus niruri* (stone breaker) appears to have some efficacy in reducing stone burden in kidney stone patients, but it needs re-evaluation in larger-scale clinical studies to better assess its true efficacy in this regard. Analysis of internet search behaviors suggests that public interest for it within the United States is growing, exceeding that for conventional surgical interventions for stone disease in recent years, possibly suggesting a growing public demand. A better understanding of its current and projected utilization in kidney stone treatment is needed. □

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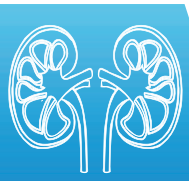
Original Research



UROLOGY

Evaluation of the efficacy of *Phyllanthus niruri* standardized extract combined with magnesium and vitamin B6 for the treatment of patients with uncomplicated nephrolithiasis

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Abstract

Introduction. The aim of our study was to assess the efficacy of Phyllanthus niruri standardized extract, combined with magnesium and B6 vitamin, used to treat uncomplicated nephrolithiasis.

Methods. We included in the present study 48 patients with uncomplicated nephrolithiasis, with the maximum calculi diameter of up to 15 mm, confirmed by non-contrast-enhanced computer tomography. Each patient followed a three-month therapeutic regimen with the above mentioned combination, with imaging assessment of the calculi after treatment.

Results. Per patient: The mean age of the patients was 48 years. The median number of calculi was 1 and the mean dimension was 5.5 mm. The stone-free status after treatment was not correlated with gender (p=0.7), side location (p=0.8) or with the number of calculi (p=0.3), but we found a correlation with the location in the upper or middle calyx (54.5% vs 13.8%, p=0.008) and with the maximum diameter (p=0.001). **Per stone:** 60 calculi were analyzed, 8.3% being located in the upper calyx, 36.7% in the middle and 55% in the lower one. After treatment, 40% were absent, 21.7% showed lower dimensions and 38.3% remained unchanged, with the mean reduction of 1.7 mm. We identified a cut-off value of ≤ 3 mm (AUC 0.9, CI:0.8-0.9, p<0.0001) for the prediction of stone-free status after treatment.

Conclusions. The current treatment had the highest efficacy in achieving stone-free status for patients with calculi ≤ 3 mm, located in the middle or upper calyx. A higher duration of the treatment might show improved results.

Keywords: antilithogenic, Phyllanthus, kidney calculi, triterpenes

Introduction

Nephrolithiasis is one of the most frequent urological benign pathology, being encountered in 1-15% of the patients [1]. This condition is 2-3 times more frequent in women compared to men, with a peak incidence being reported between 30 and 40 years [2]. The vast majority of renal calculi are composed of calcium oxalate, followed by uric acid and struvite [3].

Treatment options include extracorporeal shock wave lithotripsy, percutaneous nephrolithotomy, flexible or rigid ureteroscopy with laser stone fragmentation and retrieval and, in selected cases, the laparoscopic approach might be advised [4].

Even with adequate treatment, the recurrence rate varies between 22 and 51% at a mean follow-up of 2 to 7 years. The main reason for this finding is the imbalance between promoters and inhibitors of the lithogenic process. Patients with hypercalciuria, hyperuricosuria and hyperoxaluria have a higher incidence of recurrence, as well as those with hypocitraturia and hypomagesuria, both of them binding free calcium, thus inhibiting calcium oxalate nucleation [5].

Phyllanthus niruri, commonly known as "stone-breaker", is a member of *Euphorbiceae* family, being used for over 2000 years in treating urolithiasis [6]. *P. niruri* contains triterpenes, which are considered to be the main antilithogenic factor. It has been observed that the afore mentioned compound decreases the urinary excretion of oxalate and calcium near their normal concentration, while interfering with the glycosaminoglycans found in the matrix

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of the precipitating crystals, making them smoother and more fragile [7-9]. Another noticeable effect is that calcium oxalate crystals remain equidistantly dispersed in urine treated with *P. niruri* extract [10].

The aim of this study was to assess the efficacy of *Phyllanthus niruri* standardized extract, combined with magnesium and B6 vitamin, for the treatment of uncomplicated nephrolithiasis.

Patients and methods

Patient selection

The current study included 48 patients who presented to our department between September and December 2017 with nephrolithiasis, initially diagnosed by abdominal ultrasound and confirmed by non-contrast-enhanced computer tomography (CT). All patients signed the written informed consent, approved by our hospital's ethics committee.

Inclusion criteria were defined as asymptomatic nephrolithiasis, with the maximum diameter of the calculus up to 15 mm and with serum and urinary assessment within the biological reference interval.

Exclusion criteria were considered hypersensitivity to any of the active principles or excipients of the compound, pregnancy and lactation, imaging evidence of ureterohydronephrosis, renal colic at admission or during the study period, liver or kidney impaired function, altered coagulation, positive urine culture, concomitant use of other antilithogenic substances, urological and non-urological malignant diseases.

Out of 48 patients, 8 of them met one of the exclusion criteria before completing 3 months of treatment: 5 stopped the treatment prior to the established due date and 3 developed renal colic. Forty patients were included in the final study database.

Study layout

All patients underwent non-contrast-enhanced CT before initializing the treatment. The identified calculi were classified according to their number, location (superior, middle, inferior calyx) and size.

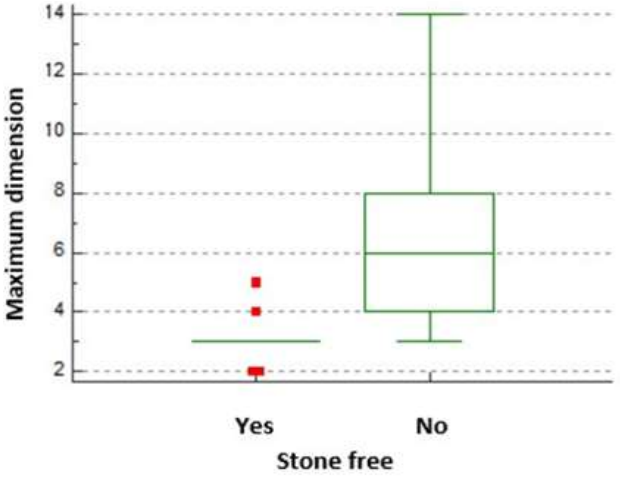

Figure 1. Stone-free status after treatment.

The treatment consisted of administration of capsules that combined three active principles, as follows: 225 mg dried Phyllanthus niruri leaf extract, 152 mg (40.5% of dietary reference intake) magnesium stearate and 2 mg (142.9% of dietary reference intake) pyridoxine hydrochloride (B6 vitamin). Used excipients were magnesium hydroxide, lactose monohydrate and talcum.

Each patient followed the same therapeutic regimen: 1 capsule twice a day, for 3 months. After treatment, the patients were reassessed by another non-contrast-enhanced CT, in order to evaluate the changes in number, location and size of the calculi.

The statistical analysis was performed using Medcalc v.12.4 (MedCalc Software bvba, Ostend, Belgium; https:// www.medcalc.org). $P<0.05$ was considered statistically significant.

Results

Per patient analysis

The study included a number of 40 patients, with the mean age of 48 years (range: 19-85 years), of whom 55% were men and 45% were female. The mean number of calculi was 1 (range: 1-4) and the mean dimension was 5.5 mm (range: 2-14 mm).

In 40% of cases, the calculi were located in the right kidney, 45% of patients had left kidney lithiasis, whereas 15% of patients had bilateral kidney lithiasis.

After treatment, a quarter of the patients were stone free. The stone free status was not correlated with the gender of the patients ($p=0.7$), with the side location ($p=0.8$) or the number of the calculi ($p=0.3$). A percentage of 13.8% of the patients with inferior calyx lithiasis achieved stone-free status after treatment, in comparison with 54.5% of the patients with calculi in the middle or superior calyx ($p=0.008$).

The maximum dimension of the calculi was correlated with achieving stone-free status ($p=0.001$) (Figure 1). The presence of a calculi equal or smaller than 3 mm had a Se of 80%, a Sp of 83.3% and an AUC of 0.887 (CI: 0.746-0.965, $p<0.0001$) for stone-free status after treatment (Figure 2).

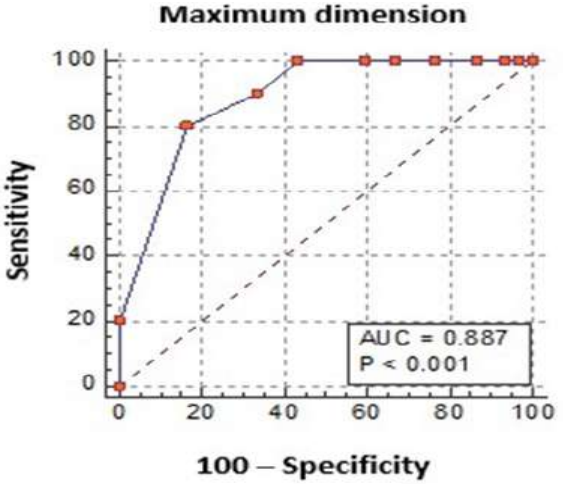

Figure 2. A cut-off value of 3 mm had a high accuracy for the prediction of stone-free status after treatment.

Per stone analysis

We analyzed a number of 60 calculi in the 40 patients included in the study. The mean dimension before treatment was 4.93 mm (range 2-14 mm). Half of the calculi were located on the right side and half on the left side. The inferior calyx harbored 55% of the calculi, whereas 36.7% were located in the middle and 8.3% in the superior calyx.

A percentage of 40% of calculi were absent on the post-treatment imaging, while 21.7% were shown with smaller dimensions. A number of 23 calculi (38.3%) had the same dimension as before the treatment (Table I).

The mean dimension of the remaining calculi after the treatment was 5.7 mm (range: 2-14 mm) and the mean reduction of the dimension was 1.7 mm (range: 1-2 mm) (Figure 3).

Using ROC curve analysis, we identified a cut-off of ≤ 3 mm (AUC 0.9, CI:0.8-0.9, p<0.0001) for the prediction of stone-free status after treatment (Figure 4). Calculi with a dimension >3 mm and <6 mm were most likely to reduce their dimension after treatment (AUC 0.6, CI: 0.4-0.7, p=0.08), whereas calculi of 6 mm or bigger would remain at the same dimension (AUC 0.83, CI: 0.7-0.9, p<0.0001). Using these cut-off values for the pre-treatment dimension of calculi, we observed a significant correlation between the dimension and response to treatment, p<0.0001.

The caliceal location of the calculi was significantly correlated with the response to treatment, as 68.18% of the calculi located in the middle calyx were absent on post-treatment imaging, in comparison with only 18% from the inferior calyx, p=0.003 (Table II).

Table I. Response to treatment stratified by the initial dimension of calculi.

Response to treatment/dimension of calculi	≤ 3 mm	> 3 mm and < 6 mm	≥ 6 mm
Absent (No of patients)	21	3	0
Reduced dimension (No of patients)	2	7	4
Identical dimension (No of patients)	4	6	13

Table II. Change in calculi diameter stratified by their caliceal location.

Response to treatment/ location of calculi	Inferior calyx	Middle calyx	Superior calyx
Absent (No of patients)	6	15	3
Reduced dimension (No of patients)	10	3	0
Identical dimension (No of patients)	17	4	2

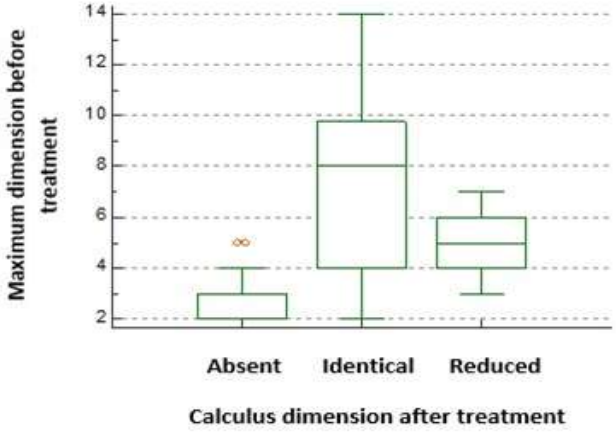

Figure 3. Calculus dimension change after treatment.

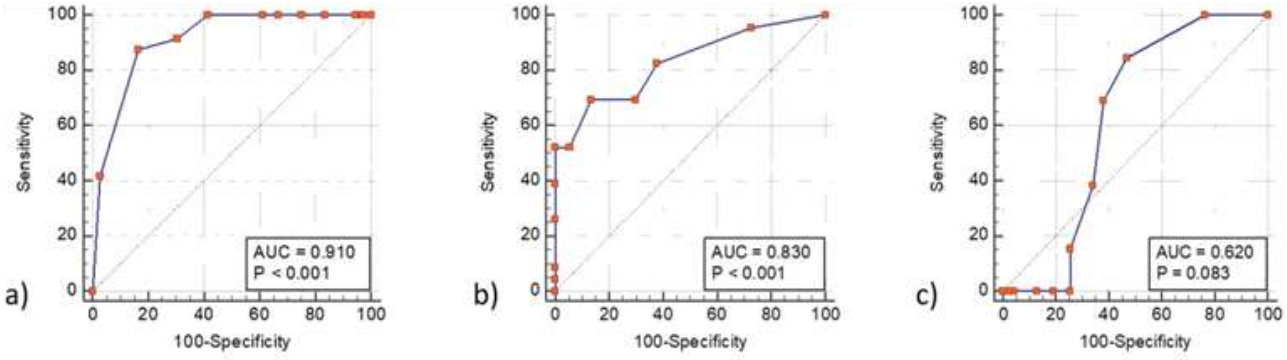

Figure 4. ROC curve analysis of the accuracy for the prediction of stone-free status after treatment depending on the initial dimension of calculi: a) ≤ 3 mm, b) 3-6mm, c) ≥ 6 mm.

Discussion

Urolithiasis remains one of the most frequent benign urological pathology, second after benign prostatic hyperplasia, with average size of calculi at diagnosis between 5 and 10 mm [11]. In our study, the mean diameter of the calculi was 4.93 mm, for which the risk of requiring a surgical intervention in the next 3 years is considered to be approximately 20%, the risk of developing renal colic is 40% and of increasing in size is 50%, as reported by Burgher et al. [12].

Phytotherapy is one of the first choices regarding pharmaceutical treatment. Micali et al. [13] analyzed the stone-free status after 3 to 6 months of treatment with dried extract of *P. niruri* in 78 patients with a history of multiple sessions of extracorporeal shock-wave lithotripsy. Average calculi diameter before treatment was 12.2 mm. The imaging assessment was done by performing abdominal X-ray and ultrasound monthly. After 6 months, 93.5% of the patients achieved stone-free status (calculi <3 mm). Response to treatment was statistically significant for calculi located in the lower calyx ($p=0.03$ for stone-free status and $p<0.001$ for fragments <3 mm), contrasting to our study, where the upper and mid calyx were the elected locations for calculi to reduce their size or to be dissolved ($p=0.003$).

Another study, conducted by Pucci et al. [14] used 4.5g of sun-dried *P. niruri* leaves, 2 times a day, for 3 months, followed by 3 months wash-out period. The study included 56 patients, with average stone size of 15.6 mm. Imaging evaluation was carried out using renal ultrasonography. After the wash-out period, mean diameter of stone fragments was 11.2 mm ($p=0.0002$). The size of the calculi had decreased in 67.8% of patients, remained unchanged in 17.8% and increased in 14.3%, in comparison with 40% of calculi being absent in our group and 21.7% showing lower dimensions. The difference in the dimension might be as a result of the imaging used, CT being the most accurate for urinary lithiasis in comparison with ultrasound or abdominal X-ray.

Other plants have proven their efficacy for the treatment of urolithiasis. Singh et al. [15] studied the effects of *Dolichous biflorus*, *Fabaceae family*, commonly known as horse gram. Forty-five patients were included. First group, 24 patients, with mean calculus size of 5.42 mm, identified by abdominal X-ray or ultrasound, were given 1-2 g/day of dried extract for 6 months. Mean diameter measured 4.26 mm. The second group comprised 21 patients, with mean calculus size of 6.46 mm, which was given 10 to 15 ml of potassium citrate six hourly, for 6 months. After treatment, the stone size dropped to 4.64 mm, but it was not considered a statistically significant change ($p>0.05$).

Celosia argentea, *Amaranthaceae* family (silver cock's comb), has ruled out potassium citrate in a clinical study performed by Rana Singh et al. [16]. Twenty-one patients received 10 mg/kg dried seed extract three times a day, compared to 23 patients receiving 0.25 mL/kg potassium citrate, 4 times a day for 6 months. The calculi were assessed using abdominal X-ray or renal ultrasound every three months. Intragroup analysis showed a significant reduction of the lithiasis in the first group (6.47 vs 3.9 mm, $p<0.05$), but not for the second one (6.46 vs 4.04, $p>0.05$). Intergroup evaluation was also statistically significant ($p>0.05$).

Although previous studies did not favor potassium citrate therapy, results may come with a longer period of time. Lojanapiwat et al. [17] studied potassium citrate therapy vs. placebo in 76 treated patients (39 and 37, respectively). All of them underwent extracorporeal shock-wave lithotripsy or percutaneous nephrolithotomy, being stone-free or with fragments <4 mm at the beginning of the study. The average stone size was 2.54 and 2.65 mm, respectively. The treated group received 27mEq potassium-sodium citrate, three times a day, for 1 year. Plain abdominal X-ray was used for evaluation. In the treated group, 92.3% of stone-free patients remained stone-free, compared to 57.7% in control one.

Soygur et al. [18] performed a similar clinical trial, targeting especially lower caliceal calculi. During 12 months, 90 patients who underwent at least one session of extracorporeal shock wave lithotripsy prior to this study were given 60 mEq potassium citrate daily. Mean reported calculus size was 9.2 mm. The stone recurrence, assessed with abdominal X-ray, was zero in the stone-free treated group, compared to 28.6% in stone-free control group. In the group with residual fragments, 45.5% of treated patients achieved the stone-free status, while the rest remained the same size, compared to untreated ones, where 12.5% were stone-free, 25% of stones remained the same and 62.5% increased in size.

In our study, the mean diameter of calculi was 4.93 mm (range 2-14 mm), similar to the studies taken into consideration. For these patients, extracorporeal shock wave lithotripsy and flexible ureteroscopy may represent an option. However, each technique associates specific morbidity rate.

Even though some self-limited side effects have been described by previously mentioned studies during phytotherapy, these were mild and did not necessitate discontinuation of treatment. Singh et al. [16] reported abdominal pain, hematuria and dysuria after using 1-2g/day *Dolichous biflorus* extract, symptoms which improved by the end of the 6-month period. Pucci et al. [14] listed similar symptoms after 6 months of 4.5g/day sun-dried *Phyllantus niruri* leaves. Micali et al. [13] used 2g/day of *Phyllantus niruri* extract, with no adverse effects to declare. A possible explanation would be the chosen pharmaceutical form, used for a lengthier period of time. In our study, no side-effects were noticed, so we consider that a lower, standardized dose might be safer and just as efficient.

Conclusion

The *Phyllanthus niruri* standardized extract administered for a 3-month period had the highest efficacy in achieving stone-free status for patients with calculi ≤ 3 mm, located in the middle or upper calyx. In patients with calculi between 3-6 mm, a 1.7 mm reduction was observed after 3 months of treatment. A higher duration of the treatment might show improved results. Other treatment options should be indicated in patients with calculi > 6 mm.

Acknowledgements

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Effect of *Phyllanthus niruri* leaves extract on urinary stone forming minerals

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Abstract-Faced with the growing evidence that stone disease is of recurrent in nature, variety of substances have been used in an effort to dissolve renal stones and to prevent recurrence. In modern medicine, allopurinol and D-penicillamine are the effective drugs for dissolution and control of recurrence of uric acid and cysteine calculus, but we hardly have any effective and useful drug for the treatment of calcium oxalate, calcium phosphate and mixed type of stones. In present work we are reporting the inhibition efficiency of *Phyllanthus niruri* leaves extract towards the mineralisation and demineralisation of calcium oxalate, calcium phosphate and calcium carbonate. We have also estimated the phosphate content of the above natural product extract. *Phyllanthus niruri* popularly known as "Stone-breaker" is a plant belonging to Euphorbiaceae family with a worldwide distribution. More than 50 compounds were identified in *Phyllanthus niruri*, including alkaloids flavanoids, lignans and triterpenes. Interestingly, given that the maintenance of normal levels of calcium is critical to the function of many plants including plant rigidity, protection, detoxification (heavy metals or oxalic acid), ion balance and even light reflection and because high cellular free calcium concentration is dangerous for these organising higher plant developed very efficient way to neutralise Ca^{2+} ions, by forming complex with oxalate.

Keywords : recurrent, calculus, mineralisation, detoxification

INTRODUCTION

Urinary stones affect 10-12% of population in industrialized countries. Their incidence has been increasing over the last years and the age of onset is decreasing. In addition, the recurrence rate is high, more than 50% after 10 years. Given that urine is naturally a super saturated solution crystalluria is often observed in normal individuals, but if crystals remain apart from each other, they are washed away by the urine flow. However under certain circumstances they bind each other due to chemical and electrical forces triggering the process of aggregation. The crystals or aggregates then attach to the epithelium, which allow them to grow further and form stone.

Alternative treatments such as the traditional herbal treatments can complement pharmacotherapies for prevention and/ or treatment of urolithiasis with less expense and fewer side effects. We have been evaluating the effects of *Phyllanthus niruri* on several stone forming minerals. Experiments studies performed by our group and others have produced interesting and hopeful data

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concerning the potential therapeutic use of *Phyllanthus niruri* to treat and/or prevent stone formation.

EXPERIMENT

Preparation of *Phyllanthus niruri* leaves extract and its acid hydrolysate

100 g of *Phyllanthus niruri* leaves was crushed and the juice was centrifuged for a few minutes and again filtered through G4 crucible. The clear filtrate obtained was used for assaying its inhibitory effect on calcium oxalate, calcium phosphate and calcium carbonate.

Now to prepare the hydrolysed extract, the filtrate was treated with about 10 ml of 2N HCl and refluxed for 2 hours. After cooling the hydrolysed extract, solid sodium bicarbonate was added with constant stirring to bring the pH back to approximately 7 and filtered through G4 crucible.

Dissolution of calcium oxalate, calcium phosphate and calcium carbonate

Known weights of calcium oxalate, calcium phosphate and calcium carbonate were suspended separately in 100 ml *Phyllanthus niruri* leaves extract (fresh/hydrolysed). The extracts were prepared in manner mentioned before. The suspension was stirred constantly for an hour on a magnetic stirrer at room temperature and then filtered through G3 crucible. The precipitate, that is undissolved salt was washed with distilled water, dried and weighted out. By subtracting the weight of undissolved salt from the initial weight taken, we get the amount of dissolved salt (Table-1).

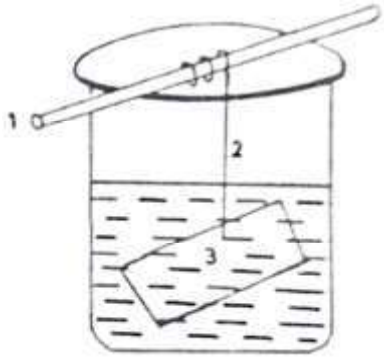
Inhibitory effect of *Phyllanthus niruri* leaves extract (fresh/hydrolysed) on the mineralisation of calcium oxalate, calcium phosphate and calcium carbonate.

The model system used to study the inhibitory effect of *Phyllanthus niruri* leaves extract (fresh/hydrolysed) on the mineralisation of calcium oxalate, calcium phosphate and calcium carbonate has some resemblance to the one (model) used by Kabra *et al.* Model consisted of two beakers of 100 ml capacity. In one of them 70 ml of 0.01 M solution of $CaCl_2$, was placed; to it 20 ml of *Phyllanthus niruri* leaves extract (fresh/hydrolysed) inhibitor was added. In the second beaker 70 ml of 0.01 M solution of sodium oxalate ($Na_2C_2O_4$) /sodium phosphate (Na_2PO_4) / sodium carbonate (Na_2CO_3) was placed and as above, 20 ml *Phyllanthus niruri* leaves extract (fresh/hydrolysed) was added to it. Two filter papers (whatman no 41,11 cm) were folded in square shape, so that both the wicks, were then suspended separately into the solution of the two beakers. Suspension was done with the help of copper wires and glass rods as shown in Fig - 1.

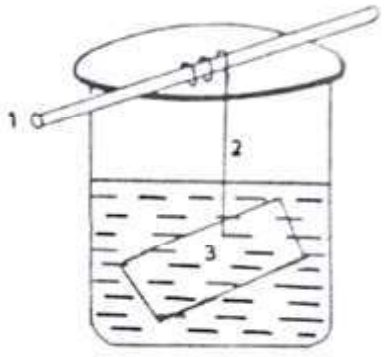
This set of two beakers was termed 'EXPERIMENTAL SET'. Next a similar assembly of two beakers with suspended filter paper wicks (weighed and noted separately) were arranged at the side of the experimental set. In this case, however, the contents of the beakers were a little different from the one in the experimental set. One of the beakers of this set contained 90 ml of 0.01 M solution of $CaCl_2$ and the other beaker contained 90 ml of 0.01 M $Na_2C_2O_4$/Na_3PO_4/Na_2CO_3 solution. No inhibitor was added in this case. This set was termed 'BLANK SET'.

APPARATUS SET-UP

1. Glass Rod 2. Copper wire 3. Wick

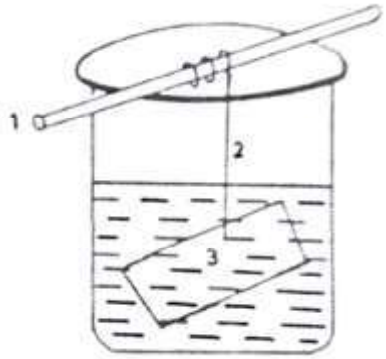


70 ml of $CaCl_2$ Solution + 20 ml of distilled water

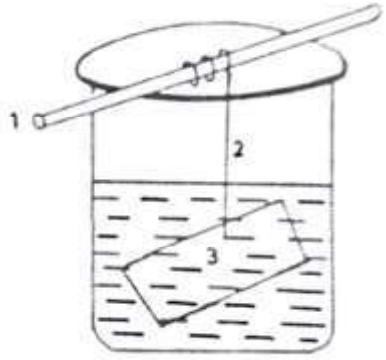


70 ml of $Na_2C_2O_4$/ Na_3PO_4/Na_2CO_3 Solution+20ml of distilled water

Blank Set



70 ml of $CaCl_2$ Solution + 20 ml of inhibitor



70 ml of $Na_2C_2O_4$/ Na_3PO_4/ Na_2CO_3 Solution + 20ml of inhibitor

Experimental Set

Fig-1

Next, the two filter paper wicks suspending in the two beakers of 'Experimental set' were interchanged at the end of every five minutes interval. This meant that the filter paper wick suspending in $CaCl_2$ solution were transferred to $Na_2C_2O_4$/ Na_3PO_4/ Na_2CO_3. solution. This interchange of wicks at the end of every five minutes was continued for four hours.

Similarly, the filter paper wicks suspending in the beakers of 'BLANK SET' were also interchanged at the end of every five minutes for four hours. Work was carried out at room temperature. At the end of the experimentation, the filter paper wicks of 'experimental set' as well as blank set after washing, were dried at 90°C in an air oven, cooled to room temperature and weighed separately. From these weights of filter papers, their corresponding weights, noted from experimentation, were deducted and thus the amount of crystalloid ($Ca_2C_2O_4$/$Ca_3(PO_4)_2$/$CaCO_3$) deposited on each paper was determined. Average deposition of oxalate in the experimental set was calculated by adding the deposition on both the papers (of experimental set) and dividing it by two. Similarly average deposition on the blank set papers was also calculated out. Further the difference between the average deposition of oxalate/ phosphate/ carbonate in blank and experimental set was calculated; this was termed 'net inhibition'. This net inhibition value was divided by the average amount deposited in the blank set and multiplied by 100 to get 'percentage inhibition' value.

Similar 'Experimental' and Blank' sets under the experimental conditions were run for different concentrations, viz. M/100, M/20. Inhibitor concentration was however kept the same in all the runs (Table -2, Table-3, Table - 4).

$$\text{Net Inhibition} = \frac{\text{Average deposition of } C_2O_7^{2-}/PO_4^{3-}/CO_3^{2-} \text{ in experimental set} - \text{Average deposition of } C_2O_7^{2-}/PO_4^{3-}/CO_3^{2-} \text{ in blank set}}{\text{Average deposition of } C_2O_7^{2-}/PO_4^{3-}/CO_3^{2-} \text{ in blank set}} \times 100$$

RESULT

Table 1.(a)- Solubilities of calcium phosphate, calcium oxalate and calcium carbonate in *Phyllanthus niruri* leaves extract.

Sl.no.	Natural Product Extract	Solubility mg/100 ml of extract		
		Calcium Oxalate	Calcium Phosphate	Calcium Carbonate
01.	*Phyllanthus niruri* leaves extract (fresh)	17.40	14.07	20.8
02.	*Phyllanthus niruri* leaves extract (hydrolysed)	21.64	18.50	17.5

Table 1.(b)- Inhibitory effect of *Phyllanthus niruri* extract on mineralisation of calcium oxalate/ calcium phosphate/ calcium carbonate

i.	**Contents of beaker in mineralisation of calcium oxalate**		
Experiment Set		Beaker -I	70 ml $CaCl_2$ + 20 ml inhibitor
		Beaker –II	70 ml $Na_2C_2O_4$ + 20 ml inhibitor
Blank set		Beaker –I	90 ml $CaCl_2$ solution
		Beaker –II	90 ml $Na_2C_2O_4$ solution
ii.	**Contents of beaker in mineralisation of calcium oxalate**		
Experiment Set		Beaker –I	70 ml $CaCl_2$ solution+ 20 ml inhibitor
		Beaker –II	70 ml Na_3PO_4 solution + 20 ml inhibitor solution
Blank set		Beaker –I	90 ml $CaCl_2$ solution
		Beaker –II	90 ml Na_3PO_4 solution
iii.	**Contents of beaker in mineralisation of calcium oxalate**		
Experiment Set		Beaker –I	70 ml $CaCl_2$ solution+ 20 ml inhibitor
		Beaker –II	70 ml Na_2CO_3 solution + 20 ml inhibitor solution
Blank set		Beaker –I	90 ml $CaCl_2$ solution
		Beaker -II	90 ml Na_2CO_3 solution

Table 2- Inhibitory values of *Phyllanthus niruri* extract (fresh/hydrolysed) on mineralisation of calcium oxalate. pH range- 5-7

No. of observation	Inhibitor	Strength of Salt forming solution	Accretion of Ca oxalate		Net inhibition difference (mg)	Percentage inhibition
			Blank Set	Experimental set		
1.	*Phyllanthus niruri* leaves extract (fresh)	0.01 M	567.0	449.0	98.0	17.28
2.	*Phyllanthus niruri* leaves extract (fresh)	0.05 M	396.0	290.0	106.0	26.76
3.	*Phyllanthus niruri* leaves extract (hydro)	0.01 M	578.6	415.0	127.6	22.05
4.	*Phyllanthus niruri* leaves extract (hydro)	0.05 M	412.0	193.0	219.0	29.36

Table 3- Inhibitory values of *Phyllanthus niruri* extract (fresh/hydrolysed) on mineralisation of calcium phosphate. pH range- 5-7

No. of observation	Inhibitor	Strength of Salt forming solution	Accretion of Ca oxalate		Net inhibition difference (mg)	Percentage inhibition
			Blank Set	Experimental set		
1.	*Phyllanthus niruri* leaves extract (fresh)	0.01 M	47.8	43.4	4.4	10.38
2.	*Phyllanthus niruri* leaves extract (fresh)	0.05 M	39.6	33.3	6.3	15.9
3.	*Phyllanthus niruri* leaves extract (hydro)	0.01 M	49.0	42.8	6.2	12.65
4.	*Phyllanthus niruri* leaves extract (hydro)	0.05 M	40.4	32.0	8.4	20.79

Table 4- Inhibitory values of *Phyllanthus niruri* leaves extract (fresh/ hydrolysed) on mineralisation of calcium carbonate.pH range- 5-7

No. of observation	Inhibitor	Strength of Salt forming solution	Accretion of Ca oxalate		Net inhibition difference (mg)	Percentage inhibition
			Blank Set	Experimental set		
1.	*Phyllanthus niruri* leaves extract (fresh)	0.01 M	32.0	28.0	4.0	12.5
2.	*Phyllanthus niruri* leaves extract (fresh)	0.05 M	15.0	14.0	1.0	6.6
3.	*Phyllanthus niruri* leaves extract (hydro)	0.01 M	27.6	17.8	9.8	35.5
4.	*Phyllanthus niruri* leaves extract (hydro)	0.05 M	8.2	6.7	1.5	18.3

DISCUSSION

The present study gives support to the importance of natural products containing acids and / or phosphates in the dissolution of renal calculi. To find out the exact nature of acids (i.e. whether hydroxy, amino or heterocyclic) and the type of phosphates (i.e. whether ortho, pyro, meta) is a difficult proposition, nevertheless, some approximation has been attempted. A glance at the percentage of phosphate in the natural product extracts suggests that the acid hydrolysis improves the phosphate extent. It might be due to the cleavage of more and more pd units of the proteins and their liberation of free chain phospho units. The consumed natural products most likely get hydrolysed in the digestive system.

Hydrolysates might be metabolites and these metabolites, enriched in chain phosphate content, must essentially act upon the urinary calcium (calcium dissolved oxalate/ calcium phosphate/ calcium carbonate) and keep them in state. The exact mechanism behind the dissolution/ inhibition activity of acids and pyrophosphates might be the aqueous soluble complexation; most likely the complexation by non-replacement type of chemical reactions.

Our findings in the present work suggest that the inhibitory action of *Phyllanthus niruri* leaves extracts in the mineralization and demineralization of urinary calcium (calcium oxalate/ calcium phosphate/ calcium carbonate) to be appreciable. A glance at the result shows that in the inhibition experiments, the accretion of calcium oxalate, calcium phosphate and calcium carbonate in the experimental sets is appreciably less than that in corresponding blank sets. Presence of inhibitor (i.e. the above mentioned extracts) in experimental set is responsible for the inhibition of calcium oxalate, calcium phosphate and calcium carbonate accretion. The percentage inhibition increases as the dilution of the salt forming solution increases. The percentage inhibitions are more in acid hydrolysate extract compared to their unhydrolysed extract. This is perhaps due to increased polyphosphate content in acid hydrolysate extracts. In the digestive system, where the dilution of salt forming solution would be very high, the inhibition effect of the above extracts can also be expected to be very high.

In the present work we have studied the effect of the inhibitors (natural product) in a diluted state, so as to obtain some information on their physiochemical effects although it is difficult to transfer directly the findings to the situation *in-vivo* because there might be difference between findings *in-vitro* and effects *in-vitro*. However, it can be assumed that factors affecting the inhibitor in their diluted state will also affect them in a concentrated state.

The order of solubilising capacities of extracts is more in calcium oxalate followed by calcium phosphate and calcium carbonate.

CONCLUSION

Owing to physiological toxicity of various synthetic compounds that can dissolve renal calculi of the types calcium oxalate calcium phosphate and calcium carbonate,

it is more beneficial to look into some physiologically non-toxic natural products that can dissolve calcium containing stones. With such a view in mind, we have focused our attention on some natural products viz *Phyllanthus niruri* leaves extract in the present work.

Our present findings with above natural product suggest that this is a good solubiliser of calcium oxalate, calcium phosphate and calcium carbonate. They do so by virtue of pyrophosphate and acids present in the extracts of the natural product.

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Original Article

STUDY ON *IN VITRO* ANTI-LITHIATIC ACTIVITY OF *PHYLLANTHUS NIRURI* LINN. LEAVES BY HOMOGENOUS PRECIPITATION AND TURBIDITORY METHOD

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ABSTRACT

Objective: Kidney stone is one of the most prevalent diseases worldwide and calcium oxalate has been shown to be the main component of the majority of stones formed in the urinary system of the patients. Traditional knowledge on use of medicinal plants for curing chronic diseases is proving its worth to modern society too. In this regards, study was conducted with an objective to find out the role of *Phyllanthus niruri* Linn. leaves extracts to inhibit stone formation and dissolution of already exiting stone in renal system using *in vitro* methods in the laboratory

Methods: Leaves powder was serially extracted in petroleum ether, ethyl acetate, methanol and water. *In vitro* study was conducted to assess anti-urolithiatic effect of plant for all the extracts with two standard drugs namely, Neeri and Cystone as control. Two methods, turbidity method and calcium oxalate dissolution methods were practiced to access the inhibition of stone formation and dissolution of stone crystals respectively. Microscopic study was done for the comparative evaluation of crystal density and size in each treatment in turbidity method.

Results: Water extract of plant leaves proved its potential statistically equal to the standard drug, cystone in dissolving the exiting calcium oxalate crystals. Water extract could dissolve 56.8% crystals while cystone dissolved 58.4% crystals in *in vitro* study and found statistically at par. Water extract also could inhibit up to 53.09% aggregation of calcium oxalate crystals as compared to the cystone with 76.54%. Among other extracts, methanol extract got second position in anti-lithiatic activity.

Conclusion: Common medical practice recommends use of cystone as effective medicine for preventing as well as treating renal stone which found at par with the water extract of plant leaves to inhibit the stone formation even in its crude form. Results guide us for the further detailed investigation and development of new drugs from this medicinal plant.

Keyword: - Kidney stone, Kidney diseases, Renal stone

INTRODUCTION

All over the world especially in developing countries, approximately 80% of population continues to use traditional medicine in primary medical problems. In the past decade, therefore, research has been focused on scientific evaluation of traditional drugs of plant origin. There is an urgent need to systematically evaluate the plants used in traditional medicine. Such research could lead to new drug discovery or advance use of indigenous herbal medicines for treatment. This revival of interest in plant derived drugs is mainly due to the current widespread belief that green medicine is safe and more dependable than the costly synthetic drugs many of which have adverse side effects [1].

Urolithiasis is characterized by the formation of a stone in the kidneys or urinary tracts. A large number of people, nearly 4–15% of the human populations are suffering from urinary stone problem all over the globe [2].The crystals of calcium oxalate (CaOx) are the primary constituent of more than 60% of the majority of human kidney stones; they exist in the form of CaOx monohydrate (COM) and CaOx dihydrate (COD) [3]. The pathogenesis of calcium oxalate stone formation is a multi-step process and in essence includes-nucleation, crystal growth, crystal aggregation and crystal retention. The stone formation requires supersaturated urine. Super-saturation also depends on urinary pH, ionic strength, solute concentration and complexations [4]. In spite of substantial progress in the pathophysiology and treatment of urolithiasis, there is no satisfactory drug being used in clinical therapy. Endoscopic stone removal and extracorporeal shock wave lithotripsy are prohibitively costly and recurrence is quite common with these procedures [5]. Thus a drug for the prevention of this disease or its recurrence would be of great interest. *Phyllanthus niruri* Linn. (Bhui amla) has occupied an important place in Indian culture and folk medicines. It has been used in all most all the traditional systems of medicine viz., *Ayurveda, Unanai* and *Sidha*. From the ancient time the tribal and rural people of our country commonly used this herb in treating various disorders. *P. niruri* has also been used traditionally for treating liver problems like hepatitis, elimination of mucous, kidney stones and diuretic problems [6,7,8].

Keeping above knowledge in the mind, current study was done to find out the stone formation inhibitor effect and stone dissolving effect of *P. niruri* extracts.

MATERIAL AND METHOD

Material collection and extract preparation

P. niruri leaves were collected in the month of October-November from the State Forest Research Institute, Jabalpur, Madhya Pradesh (M.P.), India and identification of the plant species was done in collaboration with Dept. of Botany, Govt. Model Science College, Jabalpur, M.P., India. Collected leaves were shade dried for 2 week and powdered using grinder. Weighed 500gm powdered material was loaded in Soxhlet extraction assembly. Extraction was done serially in petroleum ether, ethyl acetate, methanol up to 60 cycles and finally it was suspended in to the water for getting aqueous extract. All the extracts collected were first distilled and filtered with Whatman filter paper#1 in a Buchner funnel under vacuum pressure. The filtrate was evaporated using rotatory evaporator under reduced pressure to dry the material. In this way different crude extracts of *P. niruri* leaves were obtained.

For the control treatments, Poly herbal formulation, cystone and neeri were used and their stock solutions were prepared by suspending them in DMSO solution (50mg/ml) and filtered through a 0.22 mm pore size filter paper. ANOVA (Analysis of Variance) was

performed on mean data obtained on percentage inhibition in nucleation of CaOx and percentage dissolution of CaOx crystals.

***In vitro* anti-lithiatic activity test by turbidity method**

In vitro anti-lithiatic activity of the extract was tested in terms of inhibition of calcium oxalate formation by the extracts in the presence and absence of inhibitors (standard drugs and extract). The Precipitation of calcium oxalate at 37°C and pH 6.8 has been studied by the measurement of turbidity at 620nm. A spectrophotometer UV/Vis (Shimadzu 1800) was employed to measure the turbidity caused due to formation of calcium oxalate in treatments[3].The method used was similar to that described by Burns and Finlayson[9] with some minor modifications.

First of all, growth of stone nucleus *in vitro* in the absence of any inhibitor was done. For this, a volume of 1.0 ml of 0.025M $CaCl_2$ and 2ml of Tris-buffer (pH 7.4) were added in a test tube.

Then 1.0 ml of 0.025M sodium oxalate was added. Formation of the turbidity results immediately after mixing of above chemicals and then the measurement of turbidity formed (in terms of absorption at 620 nm in UV/Vis spectrophotometer) was started immediately up to period of 10 min (600 seconds) after the mixing of the chemicals. This control experiment was done in six replications.

Absorptions were noted down and data obtained was used as the un-controlled growth of the stone nucleus for the comparison of growth in the presence of the standard drugs and plant extracts.

Table 1: Shows reduction of CaOx nucleus formation by plants extracts compared with control drugs.

Treatment Groups	Absorbance at 620nm (after 180 min)	Regression equation	Reduction in turbidity (In %)
Control	1.0	y = 0.005x + 0.041	0.0[a]
Cystone	0.26	y = 0.001x + 0.011	76.54[f]
Neeri	0.21	y = 0.001x + 0.020	81.23[f]
Petroleum ether extract	0.93	y = 0.005x + 0.014	7.12[b]
Ethyl Acetate extract	0.79	y = 0.004x + 0.018	24.95[c]
Methanol extract	0.63	y = 0.003x + 0.019	43.71[d]
Water extract	0.43	y = 0.002x + 0.013	53.09[e]
$F_{(P<0.01)}$	-		106.35
Df	-		30
$SE_{(d)}\pm$	-		0.391
$LSD_{(P<0.05)}$	-		6.28

[a,bc]..Values designated with different alphabets are significantly different from each other.

Table 2: Shows dissolution of calcium oxalate by all extracts of *Phyllanthus niruri* and Standard drugs, Cystone and Neeri.

Treatments	Absorbance At 570nm (Mean ± Sd)	Amount of Calcium in solution (mg/dl)	Reduction in Calcium (mg/dl)	CaOx Dissolution (In %)
Control	0.760±0.06[e]	1.562	-	0.0
Cystone	0.235±0.07[b]	0.650	0.9125	58.4
Neeri	0.167±0.06[a]	0.550	1.0125	64.8
Petroleum ether extract	0.620±0.05[d]	1.313	0.2495	15.9
Ethyl Acetate extract	0.638±0.09[d]	1.325	0.2375	15.2
Methanol extract	0.346±0.08[c]	0.8375	0.7250	46.4
Water extract	0.247±0.05[b]	0.6750	0.8875	56.8
$F_{(P<0.01)}$	126.47	-	-	-
df	30	-	-	-
$SE_{(d)\pm}$	0.002	-	-	-
$LSD_{(P<0.05)}$	0.061	-	-	-

Now the study was continued to know the effect of plants extracts against stone nucleus formation *in vitro.* In this experiment four sets of test tubes with 1ml of 0.025M Calcium Chloride, 2ml Tris-buffer and 1ml (50 mg/ml solution) of four plant extracts were taken. Two more sets of test tubes were prepared same as the above in which synthetic drug of the poly herbal formulation, Cystone and Neeri were administered. Then 1 ml volume of 0.025M sodium oxalate was added to each test tube. Each set was replicated six times. Immediately after the mixing of sodium oxalate, measurement of change in turbidity of the solution was done up to the period of 10 min post mixing. Inhibition in stone nucleus formation was calculated by the graphical method using the following mathematical formula:

$$\text{Inhibition \%} = \{1-[Si / Sc]\} \times 100$$

Where;

Si: slope of graph in the presence of inhibitor (drugs/extracts).

Sc: slope of without Inhibitor (Control).

Light microscopy of the crystals formed in the solution with and without the administration of the inhibitors was also done. Photographs of CaOx were taken using the objective of 40X (Plate 1).

***In vitro* anti-lithiatic activity test by calcium oxalate dissolution method**

Behind this second experiment the idea was to know the role of plant extract in dissolving the already formed stones nucleus in renal system. For this artificial calcium oxalate crystal were prepare in the laboratory by standard method [10,11,12]. Also semi permeable membrane was prepared from egg using standard methods [13,14]. 5 mg of artificially prepared calcium oxalate crystals were weighed and were mixed with 1 ml (50 mg/ml solution) of the petroleum ether extract, ethyl acetate extract, methanol extract, water extract, cystone and neeri and packed in separate egg based semi permeable membranes by suturing. Each treatment was repeated six times. Now the material packed in semi permeable membrane was allowed to suspend in separate conical flasks containing 100 ml 0.1 M Tris buffer.

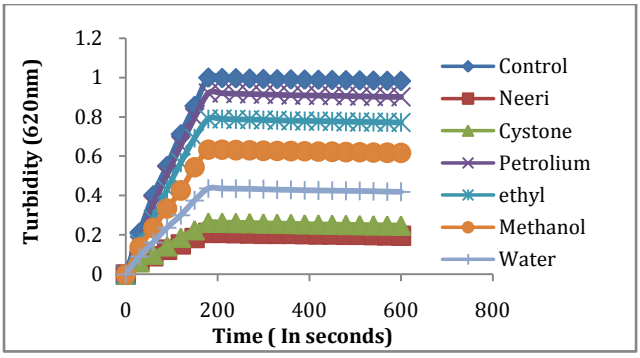

Fig. 1: Shows change in turbidity without and with plant extracts at 620nm.

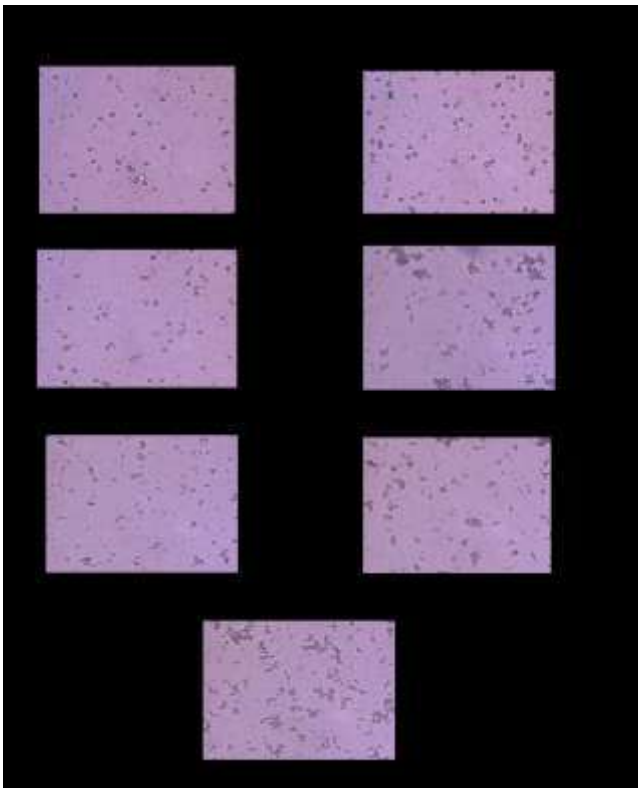

Plate 1: Shows formation of CaOx nucleus and inhibition by plant extracts and standard drugs in turbidity method.

One group served as negative control (contained only 5 mg of calcium oxalate in membrane). All the flasks were placed in incubator at 37±1^{0}C for 7 hrs. After 7 hrs, flasks with membrane were kept in magnetic stirrer for three days. After starring, membranes were taken out of the flask and content of each membrane was collected in different test tubes. Each tube then was added with 2 ml of 1 N sulfuric acid. From each test tube, 0.1ml of the suspension was taken into separate test tube and was added with 2 ml O-Cresolphthalein Complexone (O-CPC) indicator, which produces a purple color in solution[15,16]. Intensity of color produced was measured by spectroscopy method at 570 nm and using the corresponding absorption readings, concentration of calcium was determined from the standard calibration curve of calcium solution [10]. There is inverse relationship between the dissolution of calcium oxalate crystals and concentration of calcium ions in solutions.

RESULTS

In all the treatments concentration dependent initial steep rising in turbidity (nucleation) followed by decrease turbidity (aggregation) was seen. Maximum inhibition 53.09% in stone nucleus formation (turbidity) was seen by the water extract of the plant leaves after 180 seconds of chemical reaction started as compared to the control ($F_{(P<0.01)}$ = 106.3, df = 30, $SE_{(d)}$± = 0.39, $LSD_{(P<0.05)}$ = 6.28) which is significantly different from other extract. Methanol extract of the plant leaves got second position among plant leaves extracts in bio activity test which inhibited 43.71% of stone nucleus (turbidity) formation, followed by ethyl acetate extract which inhibited 24.95% in turbidity and are significantly different from each other. Least inhibition in nucleation was seen by petroleum ether extract which accounted only 7.12% inhibition. Two control drugs, Neeri and Cystone inhibited 81.23 and 76.54% in stone nucleation respectively and are significantly at par (P>0.05) to each other as shown in Table 1 and fig 1.

Photographs in Plate 1 show aggregation of calcium oxalate crystals in different treatments. Most of the nucleation and aggregation was visible in control and Petroleum ether extract treated tubes.

In Calcium oxalate dissolution study, effect of water extract was statistically equal to the effect of standard drug being used for dissolving the existing renal stone. Water extract of plant could dissolve 56.8% artificial calcium oxalate crystals packed in semi permeable and cystone could dissolve 58.4% artificial stone crystals and are statistically at par (P>0.05) ($F_{(P<0.01)}$ = 126.47, df = 30, $SE_{(d)}$± = 0.002, $LSD_{(P<0.05)}$ = 0.061). Methanol extract could dissolve 46.4% calcium oxalate crystals and got second rank among all plant extracts in dissolving effect. Petroleum ether and ethyl acetate extracts of plant leaves could dissolve 15.9 and 15.2% calcium oxalate crystals respectively and are significantly at par (P>0.05). Standard antilithiatic drug, Neeri could dissolve 64.8% of calcium oxalate crystals and was found superior over all the treatments in effect (Table 2).

DISCUSSION

Since the initial events of nucleation occurs in the first few minutes, the graphs were re-plotted within the first 3-min for each extract as well as for control[20]. In this study all the extracts of target plant leaves inhibited the stone crystal formation and dissolved the CaOx crystal also and thus proved the presence of some active antilithiatic compounds. Ability of extract to reduce the nucleation increases the metastable limit of oxalate in urine and prevents the precipitation of the CaOx crystal. Organic inhibitory compounds adsorb to the surface of the crystal, thereby inhibiting crystal nucleation, growth and aggregation [4]. Kidney stone formation is a complex process that results from a succession of several physico-chemical events including super saturation, nucleation, nucleus growth, nucleus aggregation, and retention within renal system and to prevent this physiological malfunction, variety of drugs are being used [17,18,19]. Target plants leaves have proved to have tremendous capacity to prevent the stone nucleus formation as well as extracts showed great potential to dissolve the existing stone crystal *in vitro*.

The exiting fact came out of the study is that the water extract of plant showed statistically equal potential as compared to standard drug cystone in dissolving the artificial stone crystals even in the crude form. Also the results came out of the turbidity inhibition experiment have great importance as water extract in crude form got second position in effect after the standard drugs, neeri and cystone. Up to 53.09% reduction in turbidity as compared to control and up to 56.8% crystal dissolution by the water extract is of great medical science interest.

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Evaluation of *Phyllanthus niruri* L. from Malaysia for *In-vitro* Anti-Urolithiatic Properties by Different Solvent Extraction

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Abstract. The *Phyllanthus niruri* is traditionally used for curing of kidney disorders and urinary stones in Malaysia. Hence the current work was aimed to evaluate the effect of different solvents extract (n-hexane, ethyl acetate, methanol and water) of *P. niruri* for *in vitro* anti-urolithiatic properties in terms of inhibition activity on CaOx by using the rate of CaOx aggregation assay and dissolution of calcium oxalate (CaOx) crystal by using titrimetry method. Cystone was used as positive control. The effects of cystone on slope of nucleation and aggregation as well as growth of CaOx were evaluated spectrophotometrically. The highest yield percentage of *P.niruri* was occupied by methanol (5.74 %). The maximum inhibition against aggregation of CaOx crystals was also occupied by methanol (66.67 % ± 1.61) and was comprised with alkaloid, steroid, terpenoid and tannin. Dissolution effect on calcium oxalate crystals indicates that the aqueous extracts of *P. niruri* was found to be more effective in dissolution of CaOx with 63.33 % ± 1.44. *P. niruri* significantly ($P < 0.05$) inhibited the slope of nucleation and aggregation of CaOx crystallization, and reduced the crystal density. The results of the present study confirmed that *P. niruri* leaves can be used as remedial mediator for urolithiasis. However, further studies are required for isolation and identification of active constituents and their *in-vivo* confirmation.

Keyword: crystallization, dissolution, nucleation, *P. niruri,* anti-urolithiatic.

Introduction

Urolithiasis (from Greek oûron, " urine" and " stone") is a condition in which urinary calculus is formed or located anywhere in the urinary system or stones are formed in the kidney, bladder or ureters (Sharma *et al*., 2016). Different phytochemical events begins when the formation of kidney stone occurs like crystal nucleation, aggregation and end with retention within the urinary tract. Among the several types of kidney stones, the most common are calcium oxalate representing up to 80% of the analyzed stones. Calcium containing stones may be in the form of pure calcium oxalate (50%) or calcium phosphate (5%) and a mixture of both (45%) followed by magnesium phosphate (15-20%), uric acid (10%) and cystine (1%) (Singanallur *et al*., 2017). There are numerous methods had been reported to reduced or break the kidney stone. Traditional method of treatment is being reported from plants which are the most effective. Plants based on traditional knowledge can lead to the discovery of new drug and development of pharmacologically important products for human health care (Pauzi *et al*., 2018; Subramoniam, 2014). Almost, 80% of the world's population depends on the conventional medicine to cure most of their diseases (Gul *et al*., 2019; Kennedy, 2005). There is a number of plants which show promising anti-urolithiatic activity (Ram *et al*., 2015). Nowadays, these conventional remedies have become more popular because they are very efficient, have low side effects and reduce the reformation of stone. Usually, the decoction and infusion

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methods are used for extraction that is good way for extracting compounds of various plants.

Although water decoction method is still using, even this method abundantly used huge volume of water. Moreover, there are some dis-advantages associated with water such as water that gives an excellent growth for microbes and this condition leads to microbial contamination to the samples. Moreover, it will promote hydrolysis and enzymatic degradation in the plant sample (Azmir *et al*., 2013). In addition, water also attract to an extract along with polar compounds which could obstruct in the identification and quantification (Bandar *et al*., 2013). Furthermore, the large volume of hot water usually means that the plant sample will exposure to unpleasant taste for longer period (Bone and Mills, 2013).

The *Phyllanthus niruri* is the member of the family Euphorbiaceae, and because of its speciality commonly known as as "stone-breaker" (Kieley *et al*., 2008). The habitat of the plant is moist, shady places, rock and some time epiphytes. The morphology of the plants states that leaf blade rounded to complement the elongated egg and green fruit. It can grow up to 60 cm. furthermore, the tastes of *P. niruri* is bitter, cool, and as an astringent (Dalimartha, 2008).

Wang *et al*. (1995) identified the bioactive compounds like alkaloids, coumarins flavonoids, lignans, polyphenols, saponins tannins and terpenoids from different parts of *P. niruri*. Furthermore, Bagalkotkar *et al*. (2006) stated that 50 different bio-active compounds were identified from the *P. niruri*, including flavonoids, alkaloids, triterpenes and lignans. This is the proved that this plant diverse photochemical contents in different experimental studies. Alkaloid and triterpenes reported by many research as inhibited the cytotoxicity activated by calcium oxalate (Malini *et al*., 2000). Therefore, the current study will focus on the analytical methodologies, which include the extraction and its application as anti-urolithiatic activity.

Materials and Methods

Sample collection. The grinded leaves of *P. niruri* were purchased from Seri Subah Agrofarm, Negeri Semblian, Malaysia.

Sample preparation. The grinded plant samples were kept in the room temperature and dry place to maintain them in dry condition. The moisture content of the samples were measured and maintained at consistently about not more than 10 % (Azwanida, 2015). Cystone was used as positive control while, distilled water was used as negative control.

Extraction process. The extraction method was followed by Fermeglia (2008) with slight modification. The plant samples were extracted by unlike non-polar solvents to polar solvents that are *n*-hexane, ethyl acetate, methanol and water. The extraction method was maceration using. The experiment was carried out in three replicates. The following equation used to calculate extraction yield:

$$\text{Total extract yield, Y (\%)} = \frac{\text{Total mass of extraction}}{\text{Total mass of sample}} \text{ x } 100$$

Phytochemicals analysis of the plant samples. Phyto-chemical analysis was performed by standard method followed by Tiwari *et al*. (2011). All extracts used in these assays were 1 mg/mL in concentration.

Evaluation of anti-urolithiatic properties (*in-vitro*). ***Inhibition activity of plant extracts against calcium oxalate (CaOx) crystal by aggregation assay.*** The aggregation assay was done followed by Hess *et al*. (2000) with slight modifications. In addition, the inhibition rates of CaOx aggregation by the extracts were compared with the standard drugs, Cystone. CaOx crystals solution was prepared by using 10 mM calcium chloride dihydrate and 1.0 mM sodium oxalate, containing 200 mM NaCl and 10 mM sodium acetate trihydrate. All tests were conducted at 37 °C and 5.7 pH. For crystallization of CaOx, 25 mL of calcium oxalate solution was shifted to a beaker and placed in a constantly stirring hot plate magnetic stirrer. Next to it added 1 mL of plant extract (1 mg/mL)/ Cystone (1 mg/mL)/distilled water. The formation of the turbidity results immediately after the addition of 25 mL of sodium oxalate solution. The measurement of turbidity formed in terms of absorbance at 620 nm in UV-Vis spectrophotometer. It was started continuously for ten minutes after the mixing of the chemicals. In fact, the turbidity of solution increased indicates the nucleation process, and then decreased after some time which indicates the aggregation process. This experiment was done in three replications. The percentage inhibition rate of CaOx aggregation was calculated according (Sharma *et al*., 2016).

$$\text{Inhibition \%} = [1- (Si/Sc)] \text{ x } 100$$

where;

Sc = slope of aggregation without inhibitor (negative control); Si = slope of aggregation in the presence of inhibitor (positive control/ plant extracts)

Estimation of calcium oxalate by titrimetry method. Calcium oxalate (10 mg) and plant extract or Cystone (100 mg) was weighed respectively, and packed together in the semi-permeable membrane and carefully sutured. Then, it was allowed to suspend in a conical flask containing 100 mL of 0.1M TRIS buffer. The conical flasks were kept at room temperature for seven to eight hours. The remaining contents in the semi-permeable membrane is transferred into a beaker. Next, 1N sulphuric acid (2mL) was added and titrated with $KMnO_4$ until a light pink colour appeared (Dwivedi, 2016). Consequently, 1 mL of 0.9494 N $KMnO_4$ equivalents to 0.1898 mg of calcium.

% dissolved of calcium = [(C-T)/C] x 100

where;

C = precipitate of calcium oxalate remained in control (mg); T = precipitate of calcium oxalate remained when test solution was used (mg).

Statistical analysis. All the experiments were conducted in triplicate and the data were presented as mean values and standard deviation. One way ANOVA applied on data using IBM SPSS Statistics software (Version 20.0, USA) with the level of significant $P< 0.05$.

Results and Discussion

Yields of extraction. As shown in Table 1, the effect of different solvents were studied in terms of the extraction yield. The solvents were selected based on their polarities. Polarity of a solvent plays a considerable role in the extraction process (Ahmad *et al*., 2017).

Based on the result, the highest yield percentage of P.niruri was occupied by methanol (5.74 %) followed by water (2.15%), ethyl acetate (1.46 %), and lastly n-hexane (0.98 %).

Table 1. The percentage yield of herbal plant extracts

Herbal plant	Type of solvent	Mass of sample (g)	Mass of extract (g)	Yields (%)
Phyllanthus niruri	*n*-hexane	50	0.49	0.98
	Ethyl acetate	50	0.73	1.46
	Methanol	50	2.87	5.74
	Aqueous	50	1.08	2.16

Consequently, different solvents exhibited different yield percentage for each plant samples. The results revealed that solvents yield wide range of extraction (0.98 -5.74%). Among all of the solvent used, methanol exhibited the highest percentage of yields at the maximum percentage of 5.74%. This result was similar to Kotze *et al*. (2002) which reported that methanol shown to be the best extraction solvent for Combretum erythrophyllum as compared with other extraction solvents. Similar findings have been observed in other studies done by Suleiman *et al*. (2010) which reported that hexane extract was found to be the lowest amount of extract yielded from Kirkia wilmsii.

Phytochemical associated with anti-urolithiatic properties of plant extracts. The results of phyto-chemical screening in Table 2 revealed that the presence of alkaloid, steroid, terpenoid, tannin, and saponin in plant extracts. However, based on the result obtained, the amount of detectable phytochemicals in every solvent extract is different from each other. This might be due to the different polarity of solvents could selectively extracts different type of phytochemicals (Dailey and Vuong, 2015; KV *et al*., 2014; Chavan *et al*., 2013; Rebey *et al*., 2012). Different type of phytochemicals that are present in each extract might have some positive contribution to anti-urolithiatic effect against calcium oxalate crystals either in term of inhibition or dissolution properties.

Evaluation of anti-urolithiatic properties (*in-vitro*). ***Inhibiting effect of P. niruri on calcium oxalate crystals.*** The inhibition percentage of *P.niruri* extracts was shown in Table 3. The highest inhibition percentage of *P. niruri* extract against aggregation of CaOx crystals was occupied by methanol with percentage of 66.67 % ±

Table 2. The amount of detectable phytochemical of *P. niruri* extract

Type of solvent	Alkaloid	Steroid	Terpenoid	Tannin	Saponin
n-Hexane	++	++	+	-	-
Ethyl acetate	-	++	-	-	++
Methanol	+	+++	+	+	-
Aqueous	-	+	-	+	++

+ = indicates present; '-' = indicates absent; +++ = indicates phytochemicals in high amount; ++ = indicates phytochemicals in good amount; + = indicates phytochemicals in trace but detectable amount.

1.61 and was comprised with alkaloid, steroid, terpenoid and tannin. The studies regarding the phytochemicals in *P.niruri* were proven by Calixto *et al*. (1998) and Narendra *et al*. (2012) which reported that many bio-active compound from this plant have been identified which includes alkaloids, tannin, steroids and triterpenes.

Meanwhile, the second highest percentage of inhibition was hexane extract with 53.68 % ± 2.11 which also contain the same phytochemical with methanol extract but differ in detectable amount. Moreover, aqueous and ethyl acetate extract of *P.niruri* showed quite low inhibition activity compared to methanol (29.12 % ± 1.22) and *n*-hexane (18.95 % ± 1.06). The significant different (P>0.05) between these two values was probably due to the absence of alkaloid and terpenoid in both extracts.

Dissolution of calcium oxalate crystals by titrimetry assay. This study evaluates the anti-urolithiatic activity by dissolving the artificial CaOx packed in semi permeable egg with the help of different solvent extracts of *P. niruri*. The work was performed by using *in-vitro* anti-urolithiatic model for calculating percentage dissolution of CaOx crystals. The amount of CaOx dissolved was nominated as the indicator to evaluate anti-urolithiatic activity. The results for the dissolution percentage of CaOx by plant extracts and standard are shown in Table 4. The amount of CaOx dissolved with standard drug was 73.33 % ± 3.82 which is the highest percentage as compared to plant extracts. Consequently, all extracts showed their ability to dissolve the amount of CaOx inthe range from 65.83 % to 36.67 %.

Based on the phytochemical screening, the ability of dissolving activity of plant extract on CaOx crystals could be carried out effectively with only minimum amount as compared to inhibiting activity (Fig. 1). This is in agreement with similar finding reported by Dwivedi *et al*. (2016), conclusively revealed that Colocasia leaves show good anti-urolithiatic activity by dissolving the CaOx crystals even at low amount of phytochemicals.

Table 3. The percentage of inhibition on rate of CaOx aggregation by plant extract and standard drug, cystone.

Herbal plant/ Standard drug	Type of solvent	Inhibition percentage (%) (Mean ±Standard Deviation)
Cystone	-	92.28 ± 0.61a
Phyllanthus niruri	*n*-hexane	53.68 ± 2.11d
	Ethyl acetate	18.95 ± 1.06h,i
	Methanol	66.67 ± 1.61c
	Aqueous	29.12 ± 1.22g

[a, b, c,,,] Values designated with different alphabets are significantly different from each other.

Table 4. The percentage of dissolution on CaOx crystals by plant extract and standard drugs, cystone.

Herbal plant/ Standard drug	Type of solvent	Dissolution percentage (%) (Mean ±Standard Deviation)
Cystone	-	73.33 ± 3.82a
Phyllanthus niruri	*n*-hexane	48.33 ± 3.82f,g
	Ethyl acetate	53.33 ± 5.20d,e,f,g
	Methanol	55.00 ± 0.00c,d,e,f
	Aqueous	63.33 ± 1.44b,c

[a, b, c,,,] Values designated with different alphabets are significantly different from each other.

Dissolution effect of *P. niruri* on calcium oxalate crystals. The result shows in Fig. 2. indicates that aqueous extracts of *P. niruri* was found to be more effective in dissolution of CaOx with the percentage of 63.33 % ± 1.44. This result was followed by methanol (55.00 %), ethyl acetate (53.33 % ± 5.20) and lastly *n*-hexane extract (48.33 % ± 3.82).

Similar to previous studies, aqueous extract of *Melia azedarach* was studied in male albino Wistar rats against ethylene glycol-induced nephro-lithiasis and this extract has been shown to reduce urinary calcium, oxalate, phosphate and urinary magnesium levels and urinary

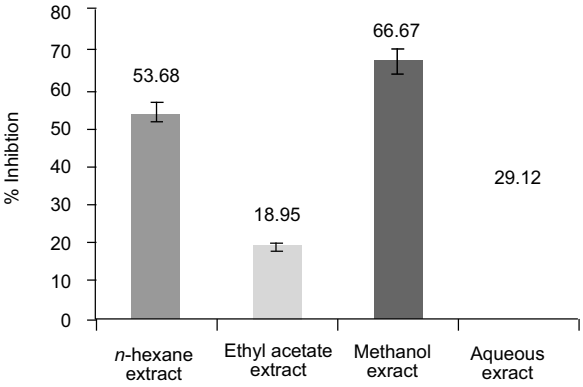

Fig. 1. CaOx inhibition activity of four solvent extracts of *P.niruri*.

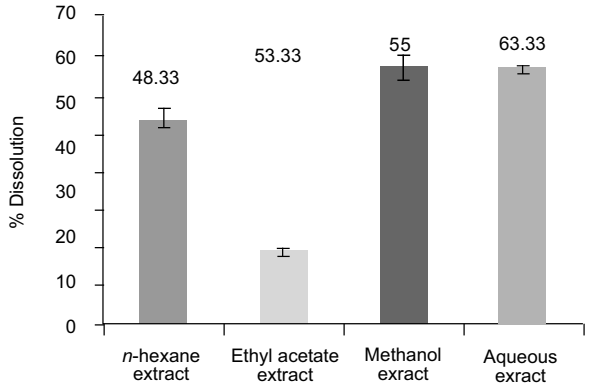

Fig. 2. CaOx dissolution activity of four solvent extracts of *P. niruri*

volume (Garimella *et al*., 2001). Moreover, the aqueous extract of C. Spiralis used at a daily dose of 0.25 and 0.5 g / Kg for 4 weeks reduced the growth of calcium oxalate calculus in the urinary bladder of rats significantly (Viel *et al*., 1999). This indicates that the aqueous type of solvent was capable of extracting various plants effectively and can positively act as anti-urolithiatic agent.

Conclusion

Based on result of extraction yield of all extracts, it has been found that the highest percentage was demonstrated of *P. niruri* extract which obtained by using methanol while the lowest yield percentage was obtained by using n-hexane as the extraction solvent. *P. niruri* extract contains different type of phytochemicals depending on the polarity of the solvent used. According to overall result of phytochemical screening, alkaloids are found to be abundant in hexane extracts while most saponins are contained in water extracts. This result might be affected by the polarity of phyto-chemicals and solvents used. Therefore, the ability of all extracts of *P. niruri* to inhibit and dissolve CaOx crystal might be beneficial in the treatment of urolithiasis in the future. However, there is a need of further scientific investigation and experimental proofs to support these preliminary findings. Besides, an additional work can also be carried out to isolate, purify and characterize bioactive compounds and to identify their possible mechanism of action.

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Conflict of Interest. The authors declare no conflict of interest.

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ORIGINAL PAPER

Effect of extract of *Phyllanthus niruri* on crystal deposition in experimental urolithiasis

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Abstract *Phyllanthus niruri* (Pn) is a plant that has been shown to interfere in the growth and aggregation of calcium oxalate (CaOx) crystals. In the present study we evaluated the effect of Pn on the preformed calculus induced by introduction of a CaOx seed into the bladder of male Wistar rats. Pn treatment (5 mg/rat/day) was initiated immediately or 30 days after CaOx seeding and thus in the presence of a preformed calculus. Animals were sacrificed 50 or 70 days after surgery. The resulting calculi were weighed and analyzed by X-ray diffraction, stereomicroscopy and scanning electronic microscopy. Precocious Pn treatment reduced the number (75%, $P < 0.05$) and the weight (65%, $P < 0.05$) of calculi that frequently exhibited a matrix-like material on its surface, compared to the untreated CaOx group. In contrast, Pn treatment in the presence of a preformed calculus did not prevent further calculus growth; rather, it caused an impressive modification in its appearance and texture. Calculi from Pn-treated animals had a smoother, homogeneous surface compared to the spicule shape of calculi found in the untreated CaOx group. XRD analysis revealed the precipitation of struvite crystals over the CaOx seed and Pn did not change the crystalline composition of the calculi. This suggests that Pn interfered with the arrangement of the precipitating crystals, probably by modifying the crystal–crystal and/or crystal–matrix interactions. Results suggest that Pn may have a therapeutic potential, since it was able to modify the shape and texture of calculi to a smoother and probably more fragile form, which could contribute to elimination and/or dissolution of calculi.

Keywords *Phyllanthus niruri* · Calcium oxalate · Struvite · Urolithiasis · Rats

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Introduction

In recent years there has been a resurgence of interest in medicinal plants that are effective, safe and culturally acceptable as an alternative treatment for many human diseases [1]. About 25% of the drugs prescribed worldwide come from plants [2]. *Phyllanthus niruri* (*P. niruri*) is a plant belonging to the Euphorbiaceae family, which has a worldwide distribution. It is used in Brazilian folk medicine by patients with urolithiasis [3, 4]. Previous clinical studies have demonstrated that *P. niruri* has no acute or chronic toxicity, and preliminary data suggest effects, which promote stone elimination in stone-forming patients, as well as the normalization of calcium levels in hypercalciuric patients [5]. Experimental studies have shown that *P. niruri* reduced the uptake of calcium oxalate crystals by MDCK cells, without evidence of cytoxicity or biochemical alterations of the

culture medium [6]. Moreover, it prevented the growth of calculi in a model of CaOx-induced urolithiasis in rats [7]. Finally it interfered with the CaOx crystallization process in vitro by reducing crystal growth and aggregation and favored the formation of a less adherent dihydrate CaOx crystalline structure [8]. Therefore, all these effects are related to the preventive potential of *P. niruri*. In the present study, we evaluated the effect of *P. niruri* treatment simultaneously or 30 days after the introduction of a CaOx seed into the bladder [9]. We also evaluated the mineral constitution and chemical composition of the stone formed with and without *P. niruri* treatment.

Materials and methods

An aqueous extract, as used in the popular medicine of *P. niruri*, was obtained from the whole plant, which was grown at the Experimental Center of the Estadual University of Campinas, São Paulo. Plant samples were cut and dried at 50°C for 2 months in a ventilated room. After drying, plants were ground in a mechanical mill and used for infusion preparation (5% w/v). The infusion was stirred for 30 min at 72°C and then vacuum filtered, concentrated, and lyophilized. *P. niruri* was administered (20 μg/g body weight/day) by gavage, diluted in 1 ml of distilled water.

CaOx seed

To prepare the CaOx seed, small disks of CaOx were obtained by a supersaturation reaction, as described previously [10]. Briefly, 100 ml of calcium chloride (0.4 mol/l) and 100 ml of potassium oxalate (0.4 mol/l) were mixed together by constant drop-wise addition to 300 ml of distilled water for 2 h with shaking at 75°C. The mixture was maintained under agitation at 75°C for an additional 5 h. Crystals were washed with distilled water to eliminate the excess of chloride and potassium, and the formed crystals were maintained in an oven at 37°C for 2 weeks to allow aggregation and to form a compact CaOx seed. The resultant material was transferred to a template containing cylinders of 4 mm diameter to obtain small disks of CaOx. Disks were weighed and sterilized before use.

Urolithiasis model

Urolithiasis was induced by introducing a CaOx disk (seed) into the bladders of adult male Wistar rats. Briefly, the bladder was exposed through a suprapubic incision under ether anesthesia and a CaOx seed was then introduced into the bladder. After suturing the bladder wall, muscle, and skin, the animals were maintained in individual cages for 24 h for observation. All animals were allowed free access to regular rat chow and tap water. The animals were then housed in cages (four animals/cage), in their respective groups (experimentals/control). The ethics committee of the São Paulo Federal University (no. 0957/02) approved the experimental protocol.

Experimental protocol

Animals were divided into six groups: C ($n = 8$): control animals, without treatment; CPn ($n = 8$): control animals treated with *P. niruri* (Pn); CaOx ($n = 5$): animals with CaOx seed with no treatment. Animals were sacrificed 50 days after the seed implantation: CaOxPn ($n = 4$): animals with CaOx seed receiving Pn treatment during 50 days starting on the day of surgery (early treatment); CaOxPn20 ($n = 5$): Pn treatment was initiated 30 days after surgery (late treatment), and lasted 20 days; CaOxPn40 ($n = 5$): Pn treatment was initiated 30 days after surgery and lasted 40 days. It was previously observed, in a pilot study, that a period of 30 days was sufficient for the development of calculi: thus, this period was adopted to start the *P. niruri* treatment. At the end of the protocol, animals were placed in metabolic cages (Nalgene, NalgeNunc Int., USA), to collect 24 h urine samples. After urine collection, animals were ether anesthetized for blood sampling (obtained from the aorta) and then killed by air injection. The bladder was exposed and the matrix calculus and satellites were removed, washed with distilled water, weighed, and stored. Biochemical determinations included urinary and plasma concentrations of sodium, potassium (photometry, Pegasus II, Tecnow, Brazil), calcium (atomic spectrophotometry), uric acid, magnesium, and creatinine (Labtest Diagnostics, Brazil).

The calculi were photographed by stereomicroscopy (Carl Zeiss, model Stemi SV11), and further analyzed by scanning electron microscopy (SEM). The compositions of calculi were obtained by X-ray diffraction (XRD) using a Siemens/Brucker model D5000 diffractometer (Germany) with Cu-Kα radiation ($\lambda = 0.15437$ nm) at 40 kW and 40 μA (step size: 0.05°θ: time per step: 1 s), and analyzed by computer program (Evaluation Diffract Plus, 2001, France).

Data are expressed as means ± standard error. Statistical analysis was carried out using ANOVA (SigmaStat for Windows version 2.0), followed by the Tukey or Dunn's tests when necessary. Parametric Student *t*-test was used when appropriate. Differences between the data were considered significant at $P < 0.05$.

Results

Biochemical analysis of blood and urine did not reveal significant alterations among groups (data not shown), thus the presence of calculus or *P. niruri* treatment did not induce detectable alterations in the animal metabolism or urinary excretions.

Figure 1 shows representative photographs of the calculi removed from the different groups. For comparison, panel A shows a CaOx seed before implantation into the bladder. The calculi removed from the untreated CaOx group consisted of a main calculus and many satellites, which were rigid and had spicule-shaped aspects (Fig. 1b). In contrast, the material (calculi) obtained from a CaOx animal treated early with *P. niruri* consisted of the CaOx seed covered by a soft material, probably an organic matrix (Fig. 1c). The animals of group CaOxPn20 (Fig. 1d) had a larger number of larger sized satellites. However, there was a clear difference in the shape of the calculi, they had a more regular surface compared with those in the untreated CaOx group.

Table 1 summarizes the data concerning number of calculi and mass. The mean mass of CaOx disks introduced into the bladder was similar among groups. As previously reported for this experimental model, the CaOx seed served as a nidus where organic and inorganic material precipitated, allowing the growth of a main calculus, and satellites. Fifty days after the introduction of CaOx seeds, the resulting calculi were, on average, 25-fold larger in mass than the original seed (0.3283 ± 0.1350 vs. 0.0131 ± 0.0004 g, $P < 0.05$). CaOx animals treated early with *P. niruri* exhibited a significant reduction in both calculus mass and satellite number compared with untreated CaOx animals. However, *P. niruri* treatment initiated after growth of calculi induced a further increase both in calculus mass (Fig. 2) and in the number of satellites.

We were surprised to find that *P. niruri* treatment initiated in the presence of preformed calculi did not minimize the growth of calculi. However, *P. niruri* clearly induced critical changes in calculus shape. These changes varied from a flattening of the edges of spicules to a complete smoothening of calculi surfaces. To observe better this effect, we next analyzed the calculi by stereomicroscopy and SEM, which allowed us to evaluate the calculi in more detail. Figure 3a shows a typical calculus and satellites grown in CaOx animals without treatment, all of them exhibited an irregular surface and a spicule shape. Group CaOxPn (Fig. 3b)

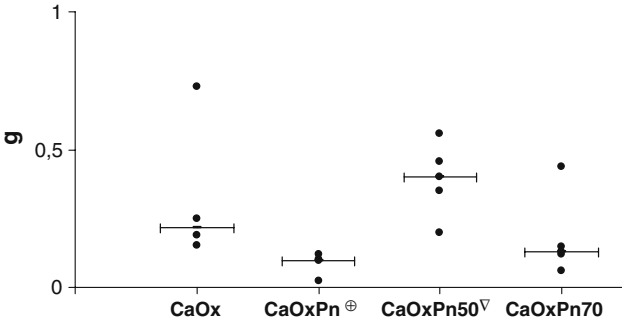

Fig. 2 Weight of calculi. Values represent the sum of the main calculus and the satellites. *Horizontal bars* indicate the median. $P < 0.05$: ⊗versus CaOx: ∇versus CaOxPn

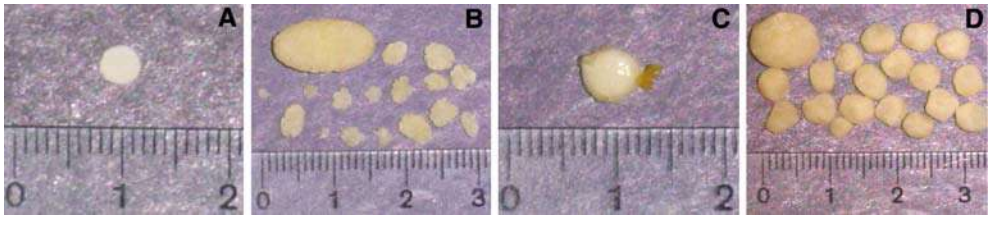

Fig. 1 Optical microscopy of the calculi. **a** CaOx seed. **b** Main calculus and satellites obtained from untreated CaOx animals. **c** Main calculus coated with an organic material from a *P. niruri* treated CaOx animal. **d** Main calculus and satellites from a CaOx animal treated with *P. niruri* in the presence of preformed calculi. The difference in calculus shape between untreated CaOx and *P. niruri* treated animals is clear

Table 1 Analyses of calculi

	CaOx seed mass (g)	Calculus mass (g)	Numbers of satellites
CaOx	0.0131 ± 0.0004	0.3283 ± 0.1350*	9.5 ± 1.6
CaOxPn	0.0131 ± 0.0002	0.0850 ± 0.0206*,***	2.5 ± 0.3**
CaOxPn20	0.0136 ± 0.0006	0.3924 ± 0.0595*	15.3 ± 1.7**,***
CaOxPn40	0.0131 ± 0.0004	0.1793 ± 0.0660*	13.0 ± 2.6***

Values represent the mean ± SE. Calculus mass corresponds to the sum of the wet main calculus and the satellites

$P < 0.05$: *versus seed weight: **versus CaOx: ***versus CaOxPn

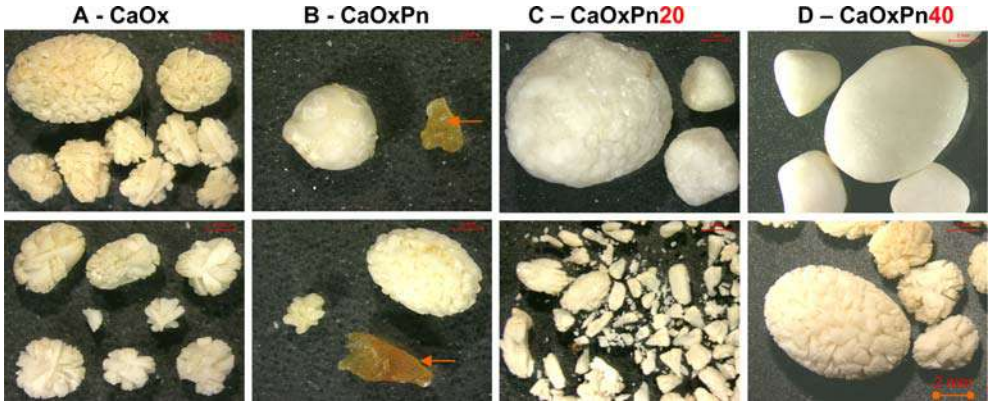

Fig. 3 Stereomicroscopy of the calculi taken from two representative animals (upper and lower panels) of each group. **a** Calculi with a homogeneous spicular shape from untreated CaOx animals. **b** Calculi from CaOx animals treated with *P. niruri* from the first day after CaOx seed implantation showing smaller calculi and very few satellites, mostly composed of an amorphous material (*arrows*). CaOx animals treated with *P. niruri* after growth of calculi for 20 (**c**) or 40 days (**d**). The variability in the response to *P. niruri* treatment is clear in (**c**). The upper panel shows calculi with a smooth surface and the lower panel shows multiple fragments of a probably more fragile calculus

exhibited almost no satellites, when present, however, they had an appearance of a predominantly organic, non-crystalline material. The calculi, when found in this group, were smaller, and had a less irregular surface compared to those found in the CaOx group with no treatment. *P. niruri* treatment initiated in the presence of a preformed calculus (groups CaOxPn20 and CaOxPn40, Fig. 3c, d, respectively) induced a clear difference in calculus surface observed with more detail under SEM (Fig. 4b, c) compared to the CaOx group (Fig. 4a). The spicule flattening or the smoother surface of calculi observed after *P. niruri* treatment on the preformed calculi can be clearly observed in Fig. 4c and d.

A summary of our XRD results is presented in Table 2. Main calculi exhibited the presence of both COM (nidus) and struvite (precipitated material) and the satellites presented only struvite.

Figure 5 shows a main calculus from an untreated CaOx animal, which was fragmented. A different material (struvite) was deposited on the CaOx seed, which acted as a central nidus.

Discussion

The experimental model of urolithiasis used in the present study was induced by a vesical foreign body (CaOx seed) implantation method that was obtained with no significant metabolic or systemic alterations. The vesical CaOx seed acts as a supporting surface, allowing organic, and inorganic material to precipitate over the central nidus, thereby mimicking a spontaneous calculus growth. Furthermore, the functional parameters of renal function, urinary pH, and urinary solute excretions were not significantly modified in this model.

The present urolithiasis model revealed that a main calculus grew over the CaOx seed together with a variable number of satellites. Since this model utilized rats, the deposited inorganic material consisted of magnesium ammonium phosphate, resulting in struvite calculi. However, the central nidus consisted of pure CaOx crystals. This result was expected since the alkaline nature of rat urine propitiates the precipitation of struvite and not CaOx crystals [11].

Results obtained with X-ray diffraction analysis confirmed the presence of both CaOX and struvite in the main calculi, and only struvite in the satellites, this suggests that the satellites probably grew on small fragments released from the main stone and acted as struvite seeds. Moreover, struvite was the main crystalline chemical composition found in all groups, both treated and untreated with *P. niruri*, indicating that the effects of *P. niruri* on the growth of calculi were not related to alterations in the inorganic elements of the calculi.

Two important effects of *P. niruri* were found in the present study. First was its ability to prevent the growth of calculi when the treatment was initiated soon after CaOx seed implantation. Second was the drastic modification in the shape and texture of the preformed calculus and after 20 or 40 days of *P. niruri* treatment the resultant stones exhibited different features. The early *P. niruri* treatment, initiated on the first day after seed implantation, caused a significant inhibition in the growth of calculi, indicating a potential prophylactic effect of *P. niruri*. This result confirmed previous data

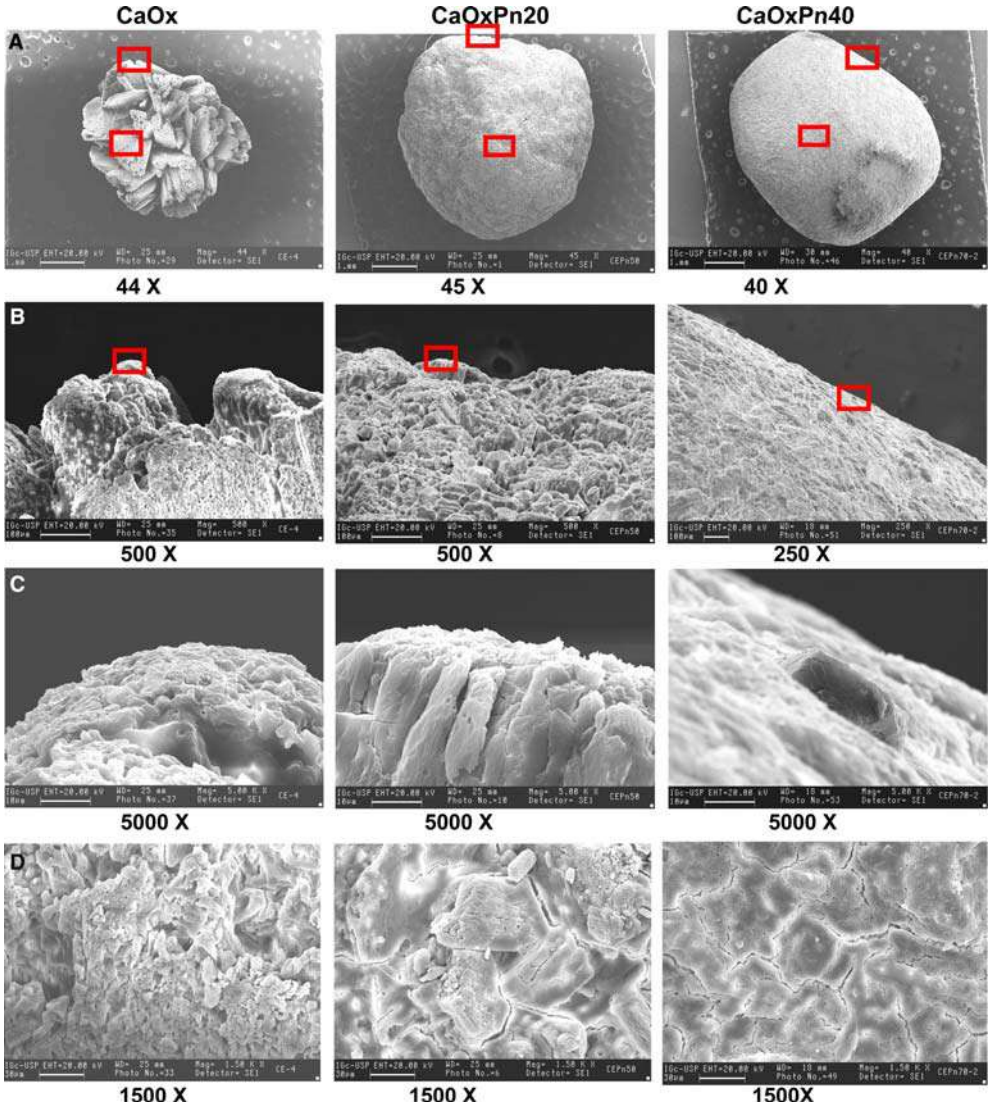

Fig. 4 Scanning electronic microscopy (SEM) of the calculi taken from CaOx untreated and late treated animals during 20 days (CaOXPn20) or 40 days (CaOxPn40). **a** Pictures show a general visualization of the calculi. *P. niruri* treated animals exhibited calculi with more regular shapes. **b** Images show a panoramic view of the calculus surface. **c** Presents the lateral view of the calculus surface in greater detail. It can be seen that the spicules are more flattened and have a smoother surface in groups treated with *P. niruri*. **d** Shows a frontal view of the calculus surface. The differential crystal deposition in calculi from *P. niruri* treated compared to untreated animals is clear

Table 2 Inorganic composition of the calculi analyzed by XRD

	CaOx	CaOxPn	CaOxPn20 satellites
COM	+	+	–
ST	+	+	+
NMM	–	+	–

ST struvite, *COM* calcium oxalate monohydrate, *NMM* non-mineral material

from our laboratory using the same animal model and experimental protocol [7] and suggests that *P. niruri* may have a potential inhibitory effect on the development of urinary calculi, probably by hindering the deposition of crystalline material on the CaOx seed. In contrast, *P. niruri* treatment initiated 30 days after CaOx seed implantation (late treatment), and thus in the presence of a preformed calculus, did not prevent the growth of further calculi, but rather induced substantial variations in the spatial arrangement of crystals, resulting in stones with a distinct shape and texture compared to untreated CaOx animals. These differences were analyzed in greater detail by SEM, the results of which revealed that an irregular precipitation of crystals was responsible for the spicule-shaped surface of the calculi taken from untreated CaOX animals. The calculi raised in *P. niruri* treated animals

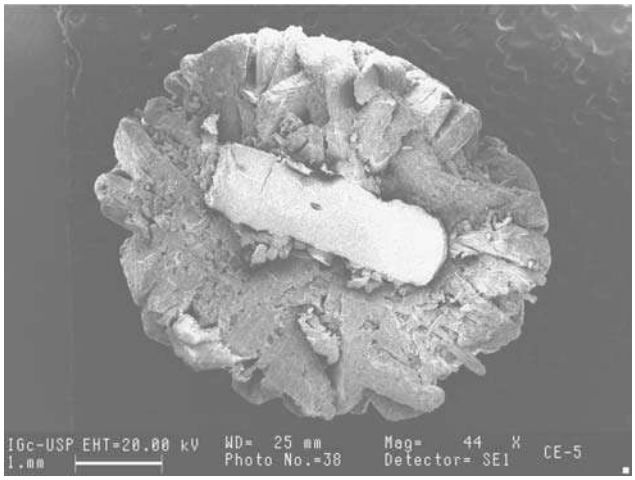

Fig. 5 SEM showing the central nidus consisting of the CaOx seed and the surrounding material formed by struvite

exhibited an anomalous crystal deposition with a filling of the spaces between the spicules, resulting in a more homogeneous and compact surface. We have previously observed that *P. niruri* interfered in the CaOx crystallization process induced in isolated human urine by reducing both crystal size and aggregation, and also by favoring the formation of a less adherent dihydrate CaOx crystalline structure [8]. Taken together, these results suggest that *P. niruri* probably interferes in the biomineralization process by promoting a different interaction between the crystal and the macromolecules of the organic matrix. It is well accepted that this interaction greatly controls crystal nucleation, size, morphology, structure, and growth rate [12–14].

The interaction between matrix proteins and crystals ensures that only crystals of a particular composition are formed, and guarantees that their various faces will grow only in certain directions to produce a structure of defined shape [15, 16]. On the other hand, the mechanisms involved in the pathological mineralization, as observed in urinary stone formation, are not well understood. However, it has become clear in recent years that the interaction between the matrix macromolecules and the nascent crystal may determine the crystal growth rate and morphology [17]. It has been demonstrated, for example, that the different isoforms of nephrocalcin induce different effects on CaOx mineralization [17]. The A and B isoforms of nephrocalcin appear to coat CaOx crystals, leaving their hydrophobic faces exposed, thereby inhibiting further mineralization. In contrast, while the C and D isoforms also coat the crystals, they promote aggregation of multiple crystals by exposing their hydrophilic face [17]. We did not analyze the organic matrix composition in the present study, however, we found in a previous study that used the same model of urolithiasis that calculi taken from CaOx animals treated with *P. niruri* from the first day after the seed implantation showed smaller and softer calculi with a higher incorporation of the matrix macromolecules glycosaminoglycans (GAGs) in the calculus structure [7]. It has been suggested that *P. niruri*-induced retardation of calculus growth was related to an increased incorporation of GAGs into the calculus, which acted to reduce the rate of calculus growth [7]. It is not presently clear if the more regular and smooth surface of the stones in animals with preexisting calculi was also the result of different matrix deposition induced by *P. niruri*.

We found some variability in both the shape and texture of calculi even among animals of the same group receiving the late treatment for 30 or 40 days. Some animals formed stones with a smooth surface (three animals), whereas others formed a less smooth surface, but flattened spicule-shaped stones and a more fragile appearance (two animals). The heterogeneity of the stones raised in this group indicates variability in the biological response to *P. niruri* treatment. Although the alterations induced by *P. niruri* were visible in all animals with preexisting calculi, further analysis of larger populations of calculi will be required to further explore this effect.

It is not clear at this time which substance(s) present in *P. niruri* would be responsible for this effect. More than 50 compounds were identified in *P. niruri*, including alkaloids, flavanoids, lignans, and triterpenes [18]. Among these substances, the triterpenes have been found to inhibit the cytotoxicity induced by calcium oxalate [19], they are also known to reduce excretion of stone forming constituents [20] and the markers of crystal deposition in the kidneys [21]. These findings point to an antilithiasic activity of these compounds, however, their effect on the growth and shape of crystals warrants further investigation.

The present study corroborates previous data suggesting the potential prophylactic effect of *P. niruri* [6–8] on the growth of calculi when the plant extract was administered before calculus development. The novel data shown here is related to the potential therapeutic effect of *P. niruri* on previously formed vesical calculi. Indeed it was clear that *P. niruri* did not prevent the growth of preexisting calculi, however, it certainly interfered with crystal deposition and substantially modified stone shape and texture. This finding raises the possibility for an alternative use of *P. niruri*, namely, to induce changes in calculi that might aid in elimination and/or dissolution of calculi.

Although the effect of *P. niruri* must be further evaluated in lithiasic patients, the results obtained in the

present study suggest that *P. niruri* may have useful therapeutic applications in patients who already have stones, while it might have a prophylactic role in persons who are at high risk but have not yet developed stones.

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