Pak. j. sci. ind. res. Ser. B: biol. sci. 2021 **64B**(1) 81-86

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

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(received January 29, 2019; revised February 21st, 2019; accepted February 26, 2019)

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

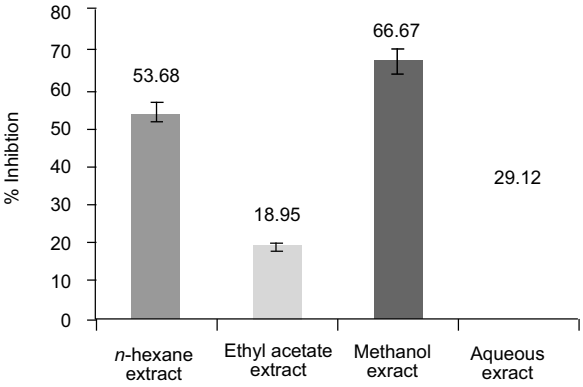

Fig. 1. CaOx inhibition activity of four solvent extracts of *P.niruri*.

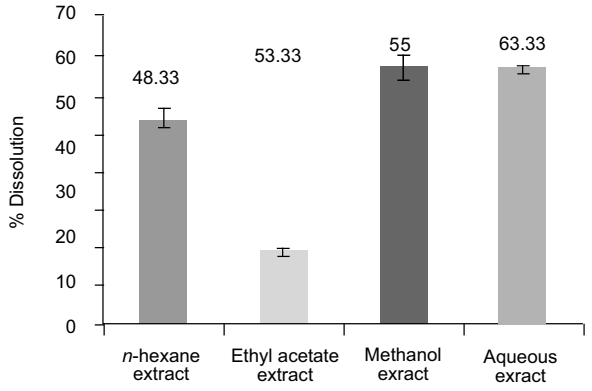

Fig. 2. CaOx dissolution activity of four solvent extracts of *P. niruri*

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.

Acknowledgement

The research is funded by the Ministry of Education Malaysia under Fundamental Research Grant Scheme (FRGS) Vot No. 1646 and partially sponsored by Universiti Tun Hussein Onn Malaysia.

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

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