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***In vitro* evaluation of anti-urolithiatic properties of *Strobilanthes crispus* extracted using different solvents**

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Abstract

Traditionally, Strobilanthes crispus is well-known for the treatment of renal diseases. The aim of the present study was to validate the traditional uses of S. crispus by evaluating its anti-urolithiatic activities in vitro. The inhibitory activity against calcium oxalate (CaOx) via aggregation assay and dissolution using titrimetric method were evaluated. The effects of S. crispus and cystone on slope of nucleation and aggregation as well as CaOx crystal growth were evaluated spectrophotometrically. S. crispus was extracted using n-hexane, ethyl acetate, methanol and water.

Methanol (5.92 %) yielded the highest percentage of extract and also showed the highest inhibitory activity against aggregation of CaOx crystals (50.54 ± 2.11 %). Ethyl acetate extract had the most effective dissolution effect on CaOx crystals (52.50 ± 2.50 %). S. crispus significantly (p < 0.05) inhibited the slope of nucleation and aggregation of CaOx crystal and reduced crystal density. The present study validated the traditional uses of S. crispus, which was found to show significant anti-urolithiatic activities. However, further studies are recommended for the isolation and identification of active constituents and their in-vivo analysis.

Keywords: Aggregation, Crystallization, Dissolution, Inhibition, *Strobilanthes crispus*, *in vitro* anti-urolithiatic.

Introduction

Urolithiasis is caused by the accumulation of inorganic salts (such as calcium, oxalate, phosphorus and ammonia) or organic salts (such as uric acid)¹⁴. Kidney stone formation has multifactorial causes including diet, genetics and environment⁹. Relatively small crystals adhere to the urothelial lining surface and then increase in size¹³.

In Malaysia and elsewhere in the world, numerous treatments have been evaluated for urolithiasis, which recurs within five years (50%). No recommended treatment can prevent recurrences²¹. The currently available clinical treatment for nephrolithiasis is either expensive or is associated with side effects. Invasive nephrolithiasis

treatment procedures can cause serious complications and also impose a financial burden on the health care system¹⁸.

In Malaysia, medicinal plants have played a prominent role in the treatment of various diseases. Plants provide a cheap source of drugs for the majority of world's population. Data from *in vitro*, *in vivo* and clinical studies show that phytotherapeutic agents can be used as an alternative or an adjunctive therapy for urolithiasis management¹.

Strobilanthes crispus is a well-known medicinal herb in Malaysia locally known as *Pecah beling* and yellow strobilanthus in English. This plant belongs to the Acanthaceae family¹². The leaves are boiled and had tea or mixed with other herbs. It is used as a folk medicine in Malaysia to treat constipation, diabetes and urolithiasis and to promote diuresis⁸. This is because this plant has many calcium carbonate cystoliths and an infusion is slightly alkaline. A high content of calcium carbonate makes this plant's boiled water slightly alkaline; this water can be used as a diuretic²⁰. This plant's pharmaceutical activity is linked to its phytochemical content.

In *S. crispus*, various phytochemical groups and components have been identified such as flavonoids, phenolic acids, alkaloids and ester glycosides³. Phenolic acids identified in the ethanol extracts of dried leaves of *S. crispus* included p-hydroxybenzoic acid, p-coumaric acid, caffeic acid, vanillic acid, gentinic acid, ferulic acid and syringic acid. Additionally, alkaloids such as caffeine and tannin are also present¹⁷. Environmental conditions have a significant effect on the phytochemical content and composition²⁴. Therefore, in the present study, different solvents were used for extraction and the anti-urolithiatic properties of the various extracts were evaluated.

Material and Methods

Sample collection: Fresh leaves of *Strobilanthes crispus* were purchased from supplier Seri Subah, Agrofarm, Negeri Sembilan, Malaysia, authenticated by Dr. Ab Rasip Bin Ab Ghani, Senior Research Officer Forest Research Institute Malaysia, Kepong 52109 Kuala Lumpur. A plant specimen was submitted to UTHM herbarium with voucher number (NYM-03-0008) for future reference. Plant samples were shade dried (25±3 °C) and then ground.

Sample preparation: The ground plant samples were kept at room temperature and in a dry place protected from moisture. The moisture content of the samples was measured and maintained consistently⁴. Cystone was used as a positive control while distilled water was used as a negative control.

Extraction process: Extraction was performed as described previously with slight modifications¹¹. The plant samples were extracted via maceration using non-polar solvents (n-hexane, ethyl acetate, methanol) and polar solvents (water). The experiment was carried out in triplicate. The following equation was used to calculate extraction yield:

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

Phytochemical analysis of plant samples: Phytochemical analysis was performed as described previously³⁰. The concentration of all extracts used in these assays was 1 mg/ml.

Evaluation of anti-urolithiatic properties (*In-vitro*)

Inhibition activity of plant extracts against calcium oxalate (CaOx) crystals by aggregation assay: The aggregation assay was performed following a previously described method with slight modifications¹⁵. In addition, the rates of inhibition of CaOx aggregation by the extracts were compared with those of the standard drug, cystone. CaOx crystal solution was prepared by using 10mM calcium chloride dihydrate and 1.0mM sodium oxalate containing 200 mM NaCl and 10 mM sodium acetate trihydrate. All tests were conducted at 37°C at 5.7 pH. For crystallization of CaOx, 25 ml of CaOx solution was transferred to a beaker and stirred on a hot plate using a magnetic stirrer.

Then, 1 ml of plant extract (1 mg/ml), cystone (1 mg/ml) or distilled water was added. The addition of 25 ml of sodium oxalate solution immediately caused the solution to become turbid. The turbidity formed was measured in terms of absorbance at 620 nm using UV-Vis spectrophotometer (U-3900H HITACHI) continuously for 10 min after the mixing of the chemicals. In fact, the turbidity of the solution increased indicating nucleation and then decreased after some time, which indicates aggregation. This experiment was performed in triplicate. The percentage inhibition rate of CaOx aggregation was calculated as follows:²⁷

$$\text{Inhibition \%} = [1 - (S_i/S_c)] \times 100$$

where S_c = slope of aggregation without inhibitor (negative control) and S_i = slope of aggregation in the presence of inhibitor (positive control/ plant extracts).

Estimation of CaOx by titrimetric method: CaOx (10mg) and plant extract or cystone (100mg) were weighed, packed together in a semi permeable membrane and carefully sutured. Then, extract was allowed to suspend in a conical flask containing 100ml of 0.1M tris buffer. The conical flasks were kept at room temperature for 7 to 8 h. The

remaining content in the semipermeable membrane was transferred into a beaker. Next, 1N sulfuric acid (2ml) was added and titrated with KMnO_4 until a light pink color appeared²⁷. Consequently, 1 ml of 0.9494 N KMnO_4 was equivalent to 0.1898 mg of calcium.

$$\% \text{ dissolved of calcium} = [(C-T)/C] \times 100$$

where C = precipitate of CaOx remained in control (mg) and T = precipitate of CaOx remained when test solution was used (mg).

Statistical analysis: All the experiments were conducted in triplicate and the data are presented as mean values and standard deviation. One-way ANOVA was applied on data using IBM SPSS Statistics software (Version 20.0, USA) and the level of significance was kept at $p < 0.05$.

Results and Discussion

Extraction yield: Extraction process is the step prior to analyzing phytochemical compounds and anti-urolithiatic properties of samples. Hence, the effect of solvents and extraction method was studied in terms of extraction yield as shown in table 1. The solvents were selected based on their polarities. Polarity of a solvent plays a considerable role in the extraction process².

Extraction with methanol led to highest yield (5.92 %) followed by ethyl acetate (1.80 %), water (1.10 %) and lastly n-hexane (0.90 %). This result was similar to that of previous studies wherein the authors reported methanol to be the best extraction solvent for *Calophyllum inophyllum* leaf extract²⁹ and grape pomace²². Similar findings have been observed in other where n-hexane extract was found to be the least effective extraction solvent for *Arisaema jacquemontii*.

Phytochemical components associated with anti-urolithiatic properties of plant extracts: Phytochemical screening revealed the presence of alkaloids, steroids, terpenoids, tannins and saponins in all extracted samples. The amount of detectable phytochemical in each extract is summarized in table 2.

In the methanolic extract, steroids and terpenoids were detected in good amount, whereas in the n-hexane extract, neither were detected. The number of detectable phytochemicals in every solvent extract is different. This might be because solvents of different polarities could selectively extract different types of phytochemicals^{6,7,25}.

Different types of phytochemicals that are present in each extract might have some positive contribution to the anti-urolithiatic effect against CaOx crystals either in terms of inhibition or dissolution properties. Similar findings have been observed in another study²⁷ which showed that the anti-urolithiatic activity of the extract was attributable to the presence of some bioactive phytochemicals

Table 1
Percentage yield of herbal plant extracts

Herbal plant	Type of solvent	Mass of sample (g)	Mass of extract (g)	Yields (%)
<i>Strobilanthes crispus</i>	n-Hexane	50	0.45	0.90
	Ethyl acetate	50	0.91	1.82
	Methanol	50	2.96	5.92
	Aqueous	50	0.55	1.10

Table 2
The amount of detectable phytochemical of each solvent

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

“+” indicates present in trace but detectable amount, “++” indicates phytochemicals in good amount; “-” absent.

Evaluation of anti-urolithiatic properties (*In-vitro*)

Inhibitory effect of *S. crispus* on CaOx crystals: Figure 1 and table 3 show that the methanol extracts of *S. crispus* show the highest percentage inhibition of CaOx at $50.54 \pm 2.11\%$. This is due to the presence of various bioactive compounds including saponins, tannins, terpenoids and steroids in the extract. Similar types of phytochemicals were found in the aqueous extract of *S. crispus* except that the amount of terpenoid and steroid was quite high. The difference in inhibition percentage value between methanol and aqueous extract might be influenced by the amount of terpenoids and steroids present in the extract. Based on another study¹⁰, the presence of terpenoids was proven to be useful in the inhibition activity against kidney stone crystals. Additionally, terpenoids blocked the formation or precipitation of CaOx, which is similar to the findings of another study²⁶ that terpenoids have the ability to reduce the size and area of CaOx formation effectively.

Furthermore, steroids in both extracts also have the potential to inhibit the rate of CaOx crystallization. This was confirmed by a previous study on the anti-urolithiatic activity of the root of *Boerhaavia diffusa* Linn. which indicated that steroid could be one of the phytochemicals that reduces the crystallization of the kidney stone⁵.

In comparison with methanol and water extract, ethyl acetate extract showed lower inhibition percentage ($23.16 \pm 2.11\%$), probably attributable to the low amount of alkaloids, terpenoids and steroids as compared to that in the other extracts. Moreover, saponins and tannins are absent in the ethyl acetate extract. Lastly, the n-hexane extract of *S. crispus* possessed the lowest inhibition percentage ($14.39 \pm 1.6\%$). The inhibitory activity of *S. crispus* extract decreased with the absence of some phytochemicals such as saponin and tannin.

Dissolution of CaOx crystals by titrimetric assay: As shown in figure 2 and table 4, the highest inhibition

percentage ($52.50 \pm 2.50\%$) was noted for the ethyl acetate extract of *S. crispus*. This extract with proven anti-urolithiatic activity was found to contain low amounts of alkaloids, terpenoids and steroids as secondary metabolites; however, this amount does not affect the dissolution activity on CaOx crystals. This was also confirmed in another study¹⁹ which reported that crude extracts of *Launea procumbens* despite having low amounts of bioactive compounds potentially dissolved CaOx crystals.

The second highest inhibition percentage was for n-hexane ($45.05\% \pm 2.20$) followed by aqueous extract ($44.50\% \pm 1.73$) and methanol ($36.67\% \pm 3.82$) extract of *S. crispus*. These extracts showed moderate dissolution effects with no significant difference ($p < 0.005$). Based on phytochemical screening, aqueous and methanol extracts contained similar type of phytochemicals including saponins, tannins, terpenoids and steroids, while the hexane extract only has alkaloids and tannins.

The difference in percentage between four solvent extracts of *S. crispus* for CaOx dissolution might be due to the presence of alkaloids. Alkaloids help improve the efficiency of ethyl acetate and hexane extract in the dissolution of CaOx crystals. Previous studies on *Bryophyllum pinnatum*²⁸, *Kalanchoe pinnata*, *Embllica officinalis*, *Bambusa nutans* and *Cynodon dactylon*⁸ reported that the medicinal and pharmacological properties including the anti-urolithiatic properties of this plant were ascribed to the presence of various phytochemicals including alkaloids. Similarly, *Phaseolus vulgaris* seed extract was found to reduce the size of CaOx monohydrate and dihydrate crystals which was attributable to the polyphenolics in the extract including alkaloids²³.

Conclusion

The ethyl acetate extract of leaves of *S. crispus* showed effective calcium stone inhibition activity as compared to the

marketed formulation cystone, whereas the methanol extract was effective against CaOx dissolution. Additional studies are needed on ethyl acetate and methanol extract of the

leaves of *S. crispus* to isolate, purify and characterize bioactive compounds and to identify their possible *in-vivo* mechanism of action.

Table 3
Percentage of inhibition of CaOx aggregation by plant extract and the standard drug cystone.

Herbal plant/ Standard drug	Type of solvent	Inhibition percentage (%) (Mean \pm Standard Deviation)
Cystone	-	92.28 \pm 0.61 ^a
<i>Strobilanthes crispus</i>	n-Hexane	14.39 \pm 1.61 ^e
	Ethyl acetate	23.16 \pm 2.11 ^d
	Methanol	50.54 \pm 2.11 ^b
	Aqueous	44.83 \pm 2.89 ^c

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

Table 4
Percentage of dissolution of CaOx crystals by plant extract and the standard drug cystone.

Herbal plant/ Standard drug	Type of solvent	Dissolution percentage (%) (Mean \pm Standard Deviation)
Cystone	-	73.33 \pm 3.82 ^a
<i>Strobilanthes crispus</i>	n-Hexane	45.05 \pm 2.20 ^{g,h}
	Ethyl acetate	52.50 \pm 2.50 ^{e,f,g}
	Methanol	36.67 \pm 3.82 ^h
	Aqueous	44.50 \pm 1.73 ^{g,h}

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

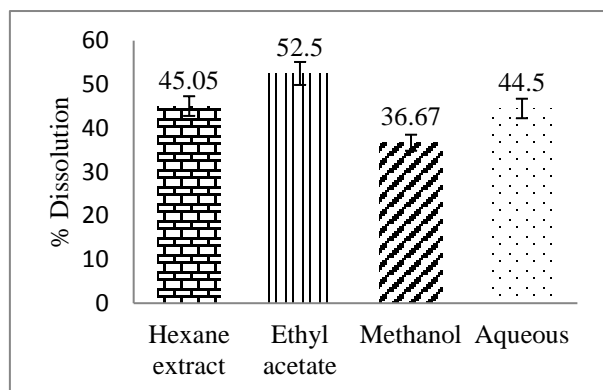


Fig. 1: CaOx inhibition activity of four solvent extracts of *S. crispus*

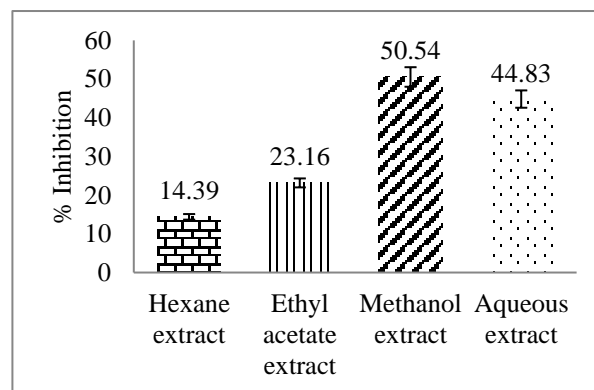


Fig. 2: CaOx dissolution activity of four solvent extracts of *S. crispus*.

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