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The effect of different solvent extraction towards antiurolithiatic properties of *Euphorbhia hirta* and *Orthosiphon stamineus*

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Abstract. There are many natural remedies derived from herbs have been used as alternatives to treat kidney stones disease effectively including *Euphorbia hirta L.* and *Orthosiphon stamineus.* Therefore, the present study aims to investigate the effect of four different extraction solvents of *E. hirta* and *O. stamineus* including hexane, methanol, ethyl acetate and water to inhibit the crystallization of calcium oxalate (CaOx) *in-vitro*. The inhibition of crystallization was studied by using rate of CaOx aggregation assay and was determined by UV-Vis spectrophotometer. Cystone was used as positive control. The result showed the best inhibition in aggregation of CaOx was exhibited by hexane extract of *E. hirta* (95.78%) which had no significant difference with Cystone, and the lowest inhibition was shown by hexane extract of *O. stamineus* (20% ±). It could be related to the presence of higher amount of alkaloid in hexane extract of *E. hirta* as compared to other extracts. In conclusion, both of plant extracts showed antiurolithiatic properties by inhibiting the crystallization of calcium oxalate. However, the rate of inhibition for both plants varies depending on the type of solvent used.

1. Introduction

Urolithiasis is a type of disease characterized by the crystallization of mineral substances within the urinary tract or kidney which commonly known as kidney stones or renal calculi [1]. The occurrence of kidney stones disease has account for 15% of world population suffered and ranked the third most common urinary tract problems following the infections of urinary tract and prostate diseases [2]. The presence of renal calculi was initiated by the interruption in the salts solubility and precipitation balance in individual's urinary tract and kidneys [3]. This is due to the changes in cycle where solid were formed from the condensation of salt in super saturation phase, starting from the nucleation of crystal, aggregation and fluid retention within urinary tract. The blockage of urine flow by kidney stone excruciated extreme pain at the abdominal area [4]. In certain circumstances, nauseous act and vomiting comes along while in severe cases, blood appeared in a person's urine if crystal size is larger than normal and was unable to pass through the tract [5].

The occurrence of kidney disease has become one of the concerns due to the worrisome increment of human population affected by it. Although this disease can be treated by using modern techniques such as Extracorporeal Shock Wave Lithotripsy (ESWL) and percutaneous nephrolithotomy, several adverse effect are bound to occur to a certain individual. This includes renal function degradation, sudden injury in renal and revival of renal calculi occurrence in kidney [6]. To combat the kidney stone disease, the antiurolithiatic plants are used as an alternative. Apart from implicating modern treatment to combat the illnesses, various types of natural remedies derived from herbs have been used effectively with the intention to eliminate and treat the kidney stones diseases. This is because medicinal plants usually contain some organic compounds such as alkaloid, steroid, terpenoid and many other phytochemicals which are beneficial for human body [7]. In addition, plant groups from Lamiaceae and Euphorbiaceae are two of the example proven and were reckon to have the ability of medicinal effect against kidney stones [8]. These plants are traditionally used to dissolve kidney stones, prevention of stone formation, ailments related to urinary stone and urinary problems [9].

O. stamineus of Lamiaceae family is a plant that resemble cat's whisker and commonly known in Australia and Southeast Asia countries consists of Malaysia, Indonesia, Thailand and other adjacent countries as Java tea and Misai Kucing [10]. Due to its mild diuretic action properties, *O. stamineus* is capable of treating kidney problems especially on the struvite stone type and bladder related problems [11]. Meanwhile, *E. hirta L*. in Malay is known as 'Ara Tanah' originates from Euphorbiaceae family [12]. Amongst traditional medicine practitioners, *E. hirta* herb was usually made into decoction or infusion to treat various diseases including intestinal parasites, diarrhea, amoebic dysentery, bronchial and respiratory diseases [13] and kidney stones [14]. In addition, the aqueous extract of this plant also can be used to treat kidney stone problem in human as diuretic agent *in-vitro* [15]. Therefore, the objective of this study was to investigate the inhibiting effect of *E. hirta* and *O. stamineus* plant extracts using different solvents towards the crystallization of calcium oxalate (CaOx) *in-vitro*.

2. Materials and methods

2.1. Collection of plant

The grinded dried leaves of *E. hirta* and *O. stamineus* were purchased from Ethno Resources Sdn Bhd, Sungai Buloh, Selangor during the month of July 2018.

2.2. Drugs and chemical

Cystone tablet (Himalaya Drug Company, India) was purchased from the Vycon Pharmacy, Kuala Lumpur. All solvents and chemicals used in this experiment consist of hexane, methanol, ethyl acetate, sodium oxalate, sodium acetate dihydrate, chloroform, Mayer's reagent, sulphuric acid, hydrochloric acid and sodium chloride were of analytical grade and obtained from QReC (New Zealand), except calcium chloride trihydrate (HmbG, Germany).

2.3. Sample preparation

The samples were dried in oven at 37°C for 5 hours, to ensure the moisture content was around 8 to 10%. This step is crucial to avoid fungal and mould contamination. Cystone was used as positive control in the evaluation of antiurolithiatic properties of plant extracts while distilled water was used as negative control.

2.4. Preparation of plant extract

The samples were extracted by maceration method using different solvents which were hexane, methanol, ethyl acetate and water. Plant sample (50 g) was weighed separately in different beakers and later was added with solvent (150 ml) respectively. It was properly sealed with aluminium foil to reduce the loss of solvent by evaporation and was left for 72 hours at room temperature with frequent agitation, until plant sample become homogenized. Next, the mixture was filtered using filter paper and the filtered

extracts were concentrated in the oven at a temperature of 40℃ to a solid form. The extracts were keep in a universal vial and stored in a freezer at -20℃ for future use.

2.5. Evaluation of antiurolithiatic properties (Aggregation assay)
The aggregation assay was done based on previous study with slight modifications [16]. Briefly, 10 mM of calcium chloride dihydrate solution and 1.0 mM of sodium oxalate solution was freshly prepared. The mixture was adjusted to pH 5.7 with the addition of 200 mM NaCl and 10 mM of sodium acetate trihydrate solution.

First, 20 ml of sodium oxalate solution was shifted into a beaker and placed on the hot plate magnetic stirrer (Favorit Model HS0707V2, Malaysia). The temperature of the mixtures was maintained at 37 °C and was continuously stirred at 800 rpm. Next, 1 ml of distilled water/standard (Cystone)/ plant extract (1 mg/ml) was added and followed by the addition of 20 ml calcium chloride solution. All experiments were performed at 37°C. The turbidity of mixture was determined by using UV Vis spectrophotometer (Thermo Scientific, BioMATE 3S, USA) at 620 nm right after the addition of solution containing calcium within 10 min times. All experiments were done in triplicate. The percentage inhibition rates of calcium oxalate aggregation containing Cystone or plant extract was collated with the control solution using the following formula [17].

$$\text{Inhibition \%} = [1- (Tsi/Tsc)] \times 100 \quad (1)$$

Where;
Tsc: slope of graph without inhibitor (negative control).
Tsi: slope of graph in the presence of inhibitor (positive control/ plant extracts).

2.6. Phytochemical screening of the extracts (Qualitative assay*)*
Phytochemical examinations were tested on all of the extracts as stated in standard methods [18]. All plant extracts used in these assays were 1 mg/ml in concentration.

2.6.1.Detection of alkaloids Plant extracts (2 ml) were dissolved individually in dilute hydrochloric acid and was filtered. Filtrates were treated with Mayer's reagent (potassium mercuric iodide). The presence of alkaloids was indicated by the formation of yellow coloured precipitate

2.6.2.Detection of saponin Plant extracts (2 ml) were diluted in 20 ml distilled water and was vigorously shaken for 15 minutes in a graduated cylinder. The presence of saponin was identified by the formation of 1 cm layer of foam.

2.6.3.Detection of steroid Plant extracts (2 ml) were flux in chloroform (2 ml) and later was added with concentrated sulphuric acid (2 ml). The presence of steroids shown by red colour produced in the lower chloroform layer.

2.6.4.Detection of terpenoid Plant extracts (2 ml) were flux in chloroform (2 ml) and left evaporated to dryness. Concentrated sulphuric acid (2 ml) was added later and heated for about 2 minutes. The appearance of a greyish colour demonstrates the existence of terpenoid.

2.7. Statistical analysis
All the experiments were conducted in triplicate and the data were presented as mean values and standard deviation. One way ANOVA were done in SPSS Statistics software (Version 20, United States) with the level of significant $p < 0.05$.

3. Result and discussion

Kidney stone disease is the third most frequent disorder of urinary system which prompted by prostate disease and infections in urinary tract [19]. The diseases related with kidney are the foremost problem for most of people due to the fact that kidney is the main excretory organ and that it is essential for normal functionality in human body [20]. In conjunction to present study related to lithiasis, four different types of solvents which are hexane, methanol, ethyl acetate and water were used to extract the bioactive compounds from *E. hirta* and *O. stamineus* plants. Both plant extracts shows the presence or absence of phytochemcial constituents like alkaloid, saponin, steroid and terpenoids as indicated in Table 1 below. Besides, the different solvents used to extract each plant sample influence the polarity of solvents and therefore could selectively extract different types of constituents due to the differences in their polarity characteristics [21].

Table 1. The phytochemicals screening for different types of solvents in *E. hirta* and *O. stamineus* plant extract.

Type of solvent	Phytochemical	Plant Extract	
		E. hirta	*O. stamineus*
Water	Alkaloid	-	-
	Steroid	++	+
	Terpenoid	-	-
	Saponin	+++	++
Hexane	Alkaloid	+++	-
	Steroid	+	+
	Terpenoid	+	+
	Saponin	-	-
Ethyl acetate	Alkaloid	+	++
	Steroid	+	-
	Terpenoid	-	-
	Saponin	-	+
Methanol	Alkaloid	-	-
	Steroid	-	-
	Terpenoid	+	+
	Saponin	+	++

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

Theoretically, the formation of such urinary calculus involves several physicochemical processes starting from nucleation, aggregation and cell growth [7]. In this study, the aggregation process of CaOx occurred in pH 5.7 condition. Then, the percentage of inhibition was calculated at the same concentration of all inhibitors. The obstruction of calcium oxalate crystallization by all plant extracts can be observed as in Table 2 and were compared with standard drug, Cystone. Next, the changes in the different solution's turbidity were plotted at different time intervals which was up to 10 minutes. As a result, all plant extract and Cystone had shown the sign of inhibition for the rate of aggregation. In fact, the turbidity of solution increased indicates the nucleation process, and then decreased after some time which indicates the aggregation process [16]. Apparently, every plant extract have difference rate of aggregation therefore the percentage of CaOx inhibition become differ according to the aggregation phenomena.

Table 2. The inhibition activity of plant extracts and Cystone towards the rate of CaOx aggregation.

Sample	Inhibition percentage (%) (Mean ± SD)
Cystone	92.63±1.34[a]
Hexane extract of *E.hirta*	95.78±2.03[a]
Water extract of *E.hirta*	76.84±1.93[b]
Ethyl acetate extract of *E.hirta*	38.95±1.25[e]
Methanol extract of *E.hirta*	28.42±0.45[f]
Hexane extract of *O. stamineus*	20.00±1.59[h]
Water extract of *O. stamineus*	32.63±0.67[g]
Ethyl acetate of *O. stamineus*	60.00±0.84[c]
Methanol of *O. stamineus*	50.53±1.20[d]

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

Based on the result obtained, the percent inhibition rates of CaOx aggregation for the positive control, Cystone was found to be 92.63% ± 1.34 while the results for hexane extract of *E. hirta* showed maximum inhibition of 95.78% ± 2.03. The remarks for hexane extract of *E. hirta* showed no significance difference of inhibition (p<0.005) compared to standard drugs, Cystone and also the highest among of all extracts. The correspondent result was probably due to the presences of high amount of alkaloid in the plant extract. This attributes was due to the fact that alkaloid is one of the phytochemical families that have anti-cristallo-oxalocalcic activity in this extract [7]. This result was contradicted to hexane extract of *O. stamineus* which showed the lowest inhibition percentage with 20% ±1.59. This might due to the absence of alkaloid.

The second highest inhibition after hexane was water extract of *E.hirta* (76.84%±1.92) while water extract of *O. stamineus* showed a lower inhibtion percentage (32.63%±0.66). The difference in the value was probably influenced by the contrast amount of saponin. In regards to the antiurolithiatic effect of bioactive constituents, saponins are well known to have anti-crystallization properties which works against the CaOx by disaggregating the suspension of mucoproteins that function as crystallization promoters [22]. Similar to previous study, plants that contain saponin has high probability of having anti-crystallization mechanisms that works towards urolithiasis [23].

Next, ethyl acetate extract of *O. stamineus* also indicates good inhibition percentage with 60.00% ±0.843 but moderate inhibition in *E. hirta* with 38.69% ±1.24. Both extracts contained alkaloid but much higher amount in *O. stamineus* and it also have saponin compared to *E. hirta.* It proved that saponin and alkaloid had good properties of antiurolithic as mentioned earlier based on the difference in value of inhibition [7][22-23].

Furthermore, methanol extract of *O. stamineus* had 50.53% ±1.20 inhibition percent while *E. hirta* had 28.42% ±0.45. Both saponin and terpenoid were found in both extracts but higher saponin content in *O. stamineus.* Apart of saponin, another bioactive compound known as terpenoid was also help in blocking the formation or precipitation of CaOx. The mechanisms of inhibition was explained by observing the effect of terpenoid in *Plantago major* extract which managed to reduce the size of crystal and inhibit the area of CaOx formation effectively [24]. In addition, plants containing terpenoid are proven effective in treating kidney stone disease [25]. In addition, plants with antiurolithiatic properties should be possessing steroid compound within their system [7][26-27]. However, the direct effect was unclear and was not expressed in more comprehensive form. Despite having outstanding performances in previous research, the presence of steroid in our study appeared to be presenting only trivial changes without much alteration on the inhibition percentage of aggregation in the extracts.

Overall, *E. hirta* showed better inhibition percentage activity compared to *O. stamineus* due to the fact that detectable amount of phytochemicals in majority of *E. hirta* extracts were deemed higher than *O. stamineus.*

4. Conclusion

In conclusion, among all types of solvent tested on *E. hirta*, hexane extract had the best inhibition result with no significance difference ($p<0.05$) with standard drug, Cystone. High percentage of inhibition by hexane extract could be related with the presences of alkaloid, terpenoid and steroid. This showed that the presence of those phytochemicals might help in preventing the aggregation of CaOx more effectively. For future used in medical field, there may be a good potential for using *E. hirta* and *O. stamineus* for patients with kidney stone disease apart from receiving modern medical treatment which commonly practice in present days.

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