IN VITRO ANTIUROLITHIATIC ACTIVITY OF ETHANOLIC EXTRACT OF *DESMODIUM TRIFLORUM*

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ABSTRACT

The ethanolic extract of *Desmodium triflorum* (EEDT) was investigated for *in vitro* antiurolithiatic activity by using nucleation and aggregation methods. Phytochemical screening was also undertaken to reveal the presence of alkaloids, flavonoids, reducing sugars and sterols in the sample. The *In vitro* studies showed that the ethanolic extract inhibits aggregation, nucleation and growth of calcium oxalate crystals. The extract exhibited inhibitory action in both nucleation and aggregation assays at significant level. The results of nucleation assay were found to be significant and showed inhibition (60.39%) compared to standard cystone drug (79.59%) and in aggregation assay also showed inhibition (70.89%) compared to cystone (79.22%) at 500µgm/mL concentration. The results clearly indicated that EEDT has the capacity to inhibit the crystal formation and aggregation.

Keywords: *In vitro*, antiurolithiatic activity, urolithiasis, *Desmodium triflorum*, Cystone.

INTRODUCTION

Kidney related diseases are a major problem for both animals and humans as they are the major excretory organs[1]. Stone formation is considered as the third common affliction of the urinary tract that affects approximately 5-12% of the global population[2]. Stone formation or lithiasis is mainly of two types, urolithiasis, and nephrolithiasis. Formation of calculi in urinary bladder or ureter or any part of the urinary tract except kidney is called urolithiasis and formation of calculi in the kidney is called nephrolithiasis[3]. Stone formation mainly occurs due to the phase change, whereby the dissolved salts undergo condensation into solids because of super saturation[4]. Aphorisms of Hippocrates is the book in which the clinical significance of urolithiasis was the first described[5].

Stone formation in urinary tract mainly causes variable degree of bleeding, pain and many crystals govern the overall nature of clinical manifestation of this complaint. The nature of macromolecules included on the surface of crystals and the type of crystals formed is answered by the urinary chemistry[6]. Calcium-containing stones usually contain about 75% of all urinary calculi, which is usually present in the form of calcium phosphate and acetate and sometimes a mixture of both the compounds. It is also noted to contain trace amounts of magnesium ammonium phosphate along with uric acid and cysteine.[7]

In the present scenario, herbal medicine has gained so much importance, since they are found to be highly effective with fewer side effects and also helps in the reduction of reoccurrence of stone formation. The search for new anti-urolithiasis activity drugs from natural sources, has gained a lot of prominence. In ayurveda, drug having the ability to dissolve and disintegrate stone is referred to as “pashambhada.” Herbal extracts may prevent stone formation by diuretic activity, crystallization inhibiting activity, etc. and finally they regulate the oxalate metabolism which finally helps in reducing the reoccurrence of renal calculi[8].

Desmodium triflorum belongs to family Fabaceae (papilonaceae); it is known as three flower beggar weed in English, and it is a perennial herb[9]. The plant is commonly available in all tropical countries including India, Philippines, Indonesia and Taiwan, and the plant is generally found in grassy places, roadsides, and lawns. The flowers are found to be very much irregular, small and appearing as bright purple. Phytochemically, the plant contains genistin, ursolic acid, fucosterol, and vitexin[10]. It is a medicinally important plant, and its leaves are used in CNS stimulation convulsions as diuretic, and in diarrhoea, The plant is also reported to contain a major alkaloid hypaphorine, *N*-dimethyltryptophan betain, and choline[11].

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The leaves contain the rare alkaloid diholosylflavone, 2-*O*-glucosylvitexin[12]. Anti-inflammatory and analgesic activities of various extracts of *Desmodium triflorum* are reported[13]. In traditional medicine and folklore, it is used in the treatment of high fever[14] and to cure bone-fracture[15].

MATERIALS AND METHODS

The plant (whole herb were used in the study) specimen for the current investigation were collected from local regions of Harapanahalli, Karnataka, India and botanically identified by Prof. K. Prabhu, Pharmacognosist, SCS College of Pharmacy, Harapanahalli. A voucher specimen was deposited at the herbarium for botanical specification. (SCSCOP.Ph.Col Herb. 003/2016-17). The plant was shade dried and pulverized separately at room temperature. The powder which was obtained was subjected to successive Soxhlet extraction for 18-20 hr, and the solution was evaporated to dryness under reduced pressure. The extract was stored in air-tight bottles until further use at 4°C in a refrigerator.

Preparation of oxalate crystals

The model chosen is a classical method of oxalate crystallization with/without inhibitor because of the simplicity and satisfactory reproducibility[16]. This model helps to assess the inhibiting capacity of used chemical species. A solution of sodium oxalate (1.34g) in 100mL of 2N H_2SO_4 and calcium chloride (1.47g) in 100ml of distilled water was allowed to react in a beaker so that the calcium oxalate is precipitated, during continuous stirring. The calcium oxalate crystals which are formed are freed from traces of sulphuric acid by treatment of ammonia solution, and finally washed with distilled water and dried at a temperature 60^{0}C for 2h[17]. The growth of crystals occurs as per the following reaction:

$$CaCl_2 + Na_2C_2O_4 \rightarrow CaC_2O_4 + 2NaCl$$

Preparation of semi-permeable membrane

Eggs were purchased from farms. The apex of the eggs was punctured by a glass rod, and all the contents were removed. The empty eggs were further washed thoroughly with more amount of distilled water and placed in a beaker containing 4mL conc. HCl in 200mL distilled water. It was kept overnight for the complete decalcification of the semi-permeable membrane, and the membrane is removed very cautiously from the eggshells on the next day. The membrane was placed in small amounts of ammonia solution for neutralization of acid traces and then finally rinsed with small amounts of distilled water. It was further stored at a pH of 7-7.4 under the moistened condition in a refrigerator[18].

Nucleation assay

A solution of sodium oxalate (7.5 mmol/L) and calcium chloride (5 mmol/L) were prepared in a buffer containing NaCl 0.15 mol/L and Tris–HCl 0.05 mol/L at pH 6.5. To 100µL of plant extracts at different concentrations (100-500 µg/mL) 950 µL of calcium chloride solution was mixed at room temperature. Crystallization was immediately started by the addition of 950 µL of sodium oxalate solution, and the reactions were carried out at room temperature. The rate of nucleation was estimated by comparing the induction time of crystals in the presence of extract and control with no extract. The optical density of the solution was recorded at 620 nm after 30 min using UV-Spectrometer[19]. The percentage of inhibition is calculated by using the following formula. The results of nucleation assay is given in Table II. The optical density of the solution was recorded at 620 nm using UV- Spectrometer.

$$\% \text{ inhibition} = \frac{\text{1-Turbidity sample}}{\text{Turbidity control}} \times 100$$

Aggregation assay

Calcium oxalate (CaOx) crystals were used at a final concentration of 0.8 mg/mL, in a buffer containing NaCl 0.15 mol/L and Tris–HCl 0.05 mol/L at pH 6.5[19]. The reaction was carried out at 37^{0}C in the presence or absence of the plant extract. The percentage of aggregation inhibition was calculated by the following formula by comparing the turbidity in the presence of standard and of plant extracts (100-500 µg/mL) with that obtained in control. The results of aggregation assay is given in Table III.

$$\% \text{ inhibition} = \frac{\text{1-Turbidity sample}}{\text{Turbidity control}} \times 100$$

RESULTS AND DISCUSSION

The pharmacological evaluation includes the *In vitro* anti-urolithiasis activity of ethanolic extract of *D. triflorum* (EEDT) with the help of nucleation and aggregation assay. The percentage increase in both the methods was measured and compared with the standard drug cystone. The results revealed that the fresh ethanolic extract has antiurolithiatic activity. The fresh ethanolic extract of *D. triflorum* was investigated pharmacologically and phytochemically. The phytochemical evaluation

includes various qualitative tests that include the test for alkaloids, glycosides, flavonoids and tannins. The results are shown in Table I.

Table I: Results of phytochemical screening of ethanolic extract of *D. triflorum*

Sr. No	Chemical Constituents	*Desmodium triflorum*
1	Alkaloids	+
2	Sterols	+
3	Proteins	-
4	Tannins	+
5	Amino acid	-
6	Glycosides	+
7	Flavonoids	+
8	Reducing sugar	+
9	Fats & oils	-
10	Phenolic compounds	+
11	Vitamins	-

+ve = Present, -ve= Absent

Table II: Inhibitory effect of *D. triflorum* on Nucleation assay

Sr. No	Concentration (µg/mL)	EEDT	Standard
1	Control	0.00	0.00
2	100	28.8±2.34	43.34±2.56
3	200	32.1±0.33	51.3±0.74
4	300	34.78±1.23	63.36±0.71
5	400	46.21±1.79	66.93±0.99
6	500	60.39±0.54	79.59±1.52

Table III: Inhibitory effect of *D. triflorum* on aggregation assay

Sr. No	Concentration (µg/mL)	EEDT	Standard
1	Control	0.00	0.00
2	100	52.09±0.46	59.31±1.31
3	200	58.76±2.01	61.2±2.21
4	300	61.94±0.28	69.96±0.64
5	400	64.66±1.06	72.09±0.61
6	500	70.89±1.54	79.22±0.85

Kidney stone formation is a cascade process that results from successive physiochemical events of retention within the renal tubules, aggregation, nucleation growth and supersaturation[20]. In the present investigation, the extract was evaluated for nucleation and aggregation assay. Many medicinal plants have been used in India and elsewhere which claim effective cure of urinary stones[21]. Among the various kinds of stones identified phosphate stones formation is more in women, while calcium stones occur mainly in men[22].

Several medicinal plants contain various active chemical compounds which have an inhibitory effect in the crystallization of calcium oxalate. The decrease in urinary output causes supersaturation of urine, due to an increase in phosphate, oxalate, and calcium, leading to the formation of stones [23,24]. The increase in calcium phosphate crystals is due to urinary phosphorus[25]. Calcium oxalates are usually formed due to the high concentration of oxalates[26].

The nucleation assay was done by turbidity method, the rate of nucleation was estimated by comparing the induction time in the presence of the extract with that of control. The OD was recorded at 620nm after 30 minutes. The increase in the concentration of the plant extract inhibited the calcium oxalate crystal growth. The maximum percentage inhibition of nucleation of calcium oxalate aggregation by EEDT and cystone were found to be 60.39%, and 79.59, respectively, at 500 µg/mL.

Calcium oxalate crystal (CaOx) aggregation was determined by the spectrophotometric assay method. The crystals in solution stick together to form large particles called aggregates. The maximum percentage inhibition of aggregation associated with the EEDT and cystone were found to be 70.89% and 79.22%, respectively, at 500 µg/mL.

CONCLUSION

The plant extract inhibits nucleation, aggregation, and growth of CaOx crystals and therefore can be used for management of kidney stones. The ethanolic extract of *D. triflorum* (EEDT) has an inhibitory effect on calcium oxalate crystallization. These data suggest that the presence of antiurolithic effect is possibly due to CaOx crystal inhibition. Further preclinical and clinical studies are needed to evaluate and establish the use of this plant for antiurolithiatic activity.

The *in vitro* antiurolithiatic activity was studied as percentage inhibition of stones by nucleation, growth and aggregation assays of EEDT at 100-500 µg/mL, taking cystone tablets as standard. The results indicated that the inhibition of growth of crystals increased with increase in the concentration of the extract. Therefore, the plant possesses antiurolithiatic activity and further *in vivo* studies as well as isolation of individual compounds responsible for the activity is necessary.

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