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Comparative Studies of Phytochemicals and *In vitro* Antioxidant activity of *Tridax Procumbens* Extracted in Different Solvents and their Effect on Calcium Oxalate And Brushite under *In Vitro* Conditions

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ABSTRACT
Kidney stone is the hard deposit form in the kidney. It affects 13% and 7 % of male and female globally. The aim of the present work was to investigate the antioxidant and antiurolithiatic activity of Tridax procumbens (stem and leaves) in the extracted in the different solvents. Also study the effect of extracts on in-vitro crystallization of CaOx and brushite crystals.The plant (stem and leaves) was extracted using soxhlet apparatus in various solvents such as toluene, ether, ethanol, aqueous flavonoid rich extract by hot percolation method. The extracts were screened for antioxidant action by DPPH, ABTS, lipid peroxidation inhibition, xanthine oxidase, Superoxide radical scavenging assay. The findings concluded that flavonoid rich extract possesses the greater inhibition on nucleation of calcium oxalate as compared to other extract-derived fractions of aerial parts and roots. Flavonoids and phenolics chiefly exhibit antioxidant activity and hence, can serve as antiurolithiatic agents.
Keywords: *Tridax procumbens, Antioxidant activity, calcium oxalate, antiurolithiatic activity, total phenolic content, total flavonoid content*

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INTRODUCTION

The hard deposit of that form in the kidney termed as renal stone or kidney stone. When it enters in the ureter termed as ureteral stone. The occurrence of kidney stone is 12 % of total population. The occurrence of disease in male and female was found to be 13 % and 7 % respectively [1]. These stones are smaller in size carried out of the human body through the urine. The smaller size stones were removed by taking painkillers and plenty of water. Medium size stones can be removed with the help of medicines used as muscles relaxant. The large stones take longer time to exit through the ureter leads to severe pain.The stones in large size need to break in small pieces by using sound wave through minor surgery. The treatment of kidney stone involves utilization of drug and extracorporeal shock wave lithotripsy (ESWL). The recurring of disorders such as hyperoxaluria and hypercalciuria which leads to the formation of calculi can be prevent by alkali citrate and diuretic. The effectiveness by the treatment was found to be low [2].The removal of stone through extracorporeal shock wave lithotripsy and surgical endoscopy used in the treatment of kidney stone. Both the methods unable to prevent the formation of new stone (50- 80%) [3]. The utilization of wave methods causes renal injury also decrease the renal function. The treatment cost was also high. The awareness about the potential benefits of herbal drugs in the treatment of kidney stone was growing. The present study involved the utilization of herbal plant *Tridax procumbens*. The common name of *Tridax procumbens* is Ghamara, also termed as coat buttons. The plant *Tridax procumbens* belongs to family Asteraceae. It is mostly prescribed from the practitioners of Ayurveda. The plant occurs mostly in course textured soil in tropical area. It is a small, semi prostate, annual or perennial and herbaceous creeper weed having short, hairy blade-like leaves. The stem is elongated to the height of 20-60 cm tall, branched, sparsely hairy, rooting at nodes. Flowers are tubular, yellow with hairs, inflorescence capitulum. The plant has two types flower, ray florets and disc florets. Leaves are simple, opposite, stipulate, lanceolate or ovate. 4-8 cm long, toothed margin, base wedge-shaped, shortly and petiole, hairy on both surfaces [4- 6]. The plant was studied for their phytochemical constituents and their pharmacological effects[7, 8] such as antioxidant activity [9, 10, 11], anti-

inflammatory activity [11], antibacterial activity [12- 15], repellency activity [16],vasorelaxant [17], antidiabetic activity [18], wound healing activity [19, 20], hepato-protective activity [21, 22], antifungal activity [23], antimalarial activity [24], anticancer activity [25], antimicrobial action [26], analgesic activity [27], immuno-modulatory activity [28], antilithiatic activity [29], antiobesity activity [30], blood coagulation and haemostatic activity [31], and hypotensive activity [32].

Taxonomic Classification

Kingdom: Plantae
Subkingdom:Tracheobionta
Division:Spermatophyta
Subdivision:Magnoliophyta
Class:Magnoliopsida
Subclass:Asteridae
Order:Asterales
Family:Asteraceae
Genus:*Tridax*
Species:*procumbens*

Uses

The herbal plant *Tridax procumbens* possess various medicinal values. In India the plant *Tridax procumbens* has been utilized to treat blisters and boils. The plant also used to treat ulcer and as hair tonic. The plant also useful to cure liver disorders. The gastritis as well as heartburn were cured with the plant extract [33]. The extracts also help to reduce blood pressure, cure severe diarrhea and dysentery [32, 34,35]. Hair falling can be reduced with the use of the *Tridax procumbens*. The plant used to cure respiratory related problems. In the present study, the leaves and stem of *Tridax procumbens* were extracted in the solvent such as ethanol, toluene, ether and water were utilized to determine its ability to scavenged radicals. The concentrations of leaves extract and stem extract were subjected to antioxidant assay by DPPH assay, ABTS assay, hydrogen peroxide and xanthine oxidase inhibitory action. The study also investigates the effect of all the prepared extracts on in-vitro crystallization of calcium oxalate. Crystallization was studied by using nucleation assay, growth assay and aggregation assay. The potential of all the extracts investigated against anti-lithiatic action and.

MATERIAL AND METHOD

Material

The raw drug (stem and leaves) *Tridax procumbens* was collected from the college campus. The authentication was done at St. Xavier's college Mumbai (Herbarium No: NI- 5524). The chemicals required for all testing parameters such as Methanolic solution of DPPH (1mM), Drug stock solution (5-100µg/ml), ABTS solution (2mM), Potassium Persulfate solution(17mM), Tris-buffer (40mM) solution, Potassium chloride (300mM) solution, Ammonium ferrous sulphate (0.16mM) solution, Ascorbic acid (0.06mM) solution, Thiobarbituric acid 0.8% solution, Sodium dodecyl sulphate 8.0% solution, Acetic acid glacial 20 % solution, Xanthine, Sodium phosphate buffer, DMSO EDTA, Solid Potassium superoxide, Nitrobluetetrazolium, Phosphate buffer (pH 7.4)were purchased from sigma Aldrich, India.

Methodology

Extraction process:

The stem and leaves of plant *Tridax procumbens* were collected from our college campus. The stem and leaves were cleaned and dried in shade. Both samples (leaves and stem) were powdered and then passed through the coarse sieve (0.2mm).10 gm of each sample was taken separately in a thimble and placed in a soxhlet apparatus. The extraction was by hot percolation method using 200ml of 4 different solvents including ethanol, toluene, ether and aqueous. The extraction was carried out until the plant materials become colourless. The extracts were collected and evaporated in a boiling water bath at 60°C. The residues were kept in a sterile, air tight container and stored in a refrigerator.

Determination of total phenolic content

The Folin-Ciocalteu technique was followed to estimate the total phenolic content present in the methanolic extract of plant. Gallic acid in the methanol was used as standard to draw standard curve. The determination of calibration curve was prepared by mixing 1 ml aliquots of 2, 4, 6, 8 and 10 µg/ml gallic acid solution in methanol, 5 ml of Folin-Ciocaleu reagent and 5 ml of sodium carbonate. The absorbance was measured at 765 nm after incubating the mixture for 30 min at 20°C.1 ml of methanolic extract (10mg/ml) was mixed with the Folin-Ciocaleu reagent as described above. The absorbance was measured 1 hr for the estimation of plant phenolics. All determinations were performed in triplicate. Total phenolic content in the methanolic extract of plant were calculated by the following formula:

$C = \frac{c \times V}{m}$

Where,

C = Total phenolic content (mg/gm);

c = The concentration of gallic acid established from the calibration curve(mg/ml);

V = Extract volume (ml);

m = Weight of the sample. [36]

Determination of total flavonoid content [36]

Rutin was used as standard to obtained standard curve. The estimation of total flavonoid content was carried out by mixing 2 ml of 10, 20, 30, 40, 50, and 60 |ag/ml rutin in methanolic solutions with 2 ml (20 gm/L) aluminum trichloride and 6 ml (50 gm/L) sodium acetate. The mixture was maintained at 20°C for 2.5 hrs. The absorbance was measure at 440 nm. The procedure was repeated by replacing standard solution rutin with 2 ml of plant extract (10 mg/ml). All determinations were carried out in triplicate. The content of flavonoids, in rutin equivalents (RE) was calculated by the following formula:

$$X = \frac{C \times V}{m}$$

Where,

X = total flavonoid content, mg/gm plant extract in RE;

C = the concentration of rutin solution, established from the calibration curve, mg/ml;

V = the volume of extract, ml;

m = the weight of pure plant methanolic extract, gm.

***In vitro* Antioxidant activity**

The antioxidant action of herbs was measured by various techniques. The methods are popular because of their ease, speed and sensitivity. The availability of antioxidant results in disappearance of chromogens. In the present study antioxidant action was measured through DPPH, ABTS and hydrogen peroxide method.

DPPH Assay [37, 38]

Principle: The technique is based on scavenging of the 1,1-diphenyl-2-picrylhydrazyl radical (DPPH) from the antioxidants. DPPH is the stable free radical. When the DPPH solution mixed with the extracts, the reaction of antioxidant with DPPH results in the formation of 1,1-Diphenyl-2-picryl hydrazine which do not absorb at 517 nm.

Method: The amount of DPPH (0.1mM) was mixed in methanol. From the prepared solution1 ml volume was added to 3 ml of test compounds at different concentration (5-100µg/ml).After the mixing the solutions were keptaside for 30 min. At 517 nm the absorbance of the sample was measured. The measurement of absorbance of DPPH was carried out by performing the blank experiment.

Calculation of 50 % inhibition concentration (IC50)

The optical density obtained with each concentration of selected test compounds and Butylated Hydroxyl Toluene (BHT). Percentage inhibition was calculated using equation 1. The IC50 value was determined by plotting graph taking concentration on X- axis and % inhibition on Y-axis.

$$\% \text{ Scavenging} = \frac{\text{Absorbance of control} - \text{Absorbance of test}}{\text{Absorbance of control}} \times 100$$

ABTS Assay [38]

Principle: The combination of metmyoglobin with hydrogen peroxide produced radicals of ferryl myoglobin. The oxidation of ABTS (2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) was carried out by radicals ferryl myoglobin results in the formation of ABTS.+ radical cation. The resulting mixture is green in color. The absorbance was measured at 734nm. The formation of colored ABTS radical was suppressed as the antioxidant inhibits the oxidation of radicals electron donation radical scavenging. At the absorbance 405 nm the concentration of antioxidant in the test sample is inversely proportional to the ABTS radical formation.

metmyoglobin + H_2O_2 ⟶ ferryl myoglobin + H_2O $ABTS^{+\cdot}$

ferryl myoglobin ⟶ $ABTS^{\cdot+}$ + metmyoglobin

[Antioxidants inhibit the oxidation of ABTS by electron transfer radical scavenging]

Working solution of ABTS•+

Distilled water was used to produce ABTS solution (2 mM) and potassium persulfate solution (17mM). Prepared the solutions separately kept aside for 12-16 hrs in the dark. The process leads to the formation of ABTS cation radical.

Method: With the help of the ABTS+• radical cation which are stable, the antioxidant action of all the test samples were estimated. The blue/ green color chromophore were generated when the reaction was

carried out between ABTS and potassium persulfate. The antioxidant results in the decreased in the coloration leads to decrease of absorbance at 734 nm. ABTS (7mM) was prepared by dissolving in water. The reaction between stock solution of and 2.45 mM potassium persulfate produced ABTS+. (radical cation). The mixture was allowed to stand for 12 -16 hrs in the dark room at ambient temperature. The solution was further diluted with 2 mM phosphate buffer maintained at pH 7.4 to achieve an absorbance of 0.8 ±0.014 at 734 nm. One min after the mixing of test sample and ABTS+. solution, the absorbance was recorded using UV-vis spectrophotometer at 734 nm. The blank reading was obtained by using phosphate buffer solution. The % radical-scavenging activity of the samples was determined using the formula

$$\% \text{ radical scavenging activity} = \frac{\text{A control} - \text{A sample}}{\text{A control}} \times 100$$

Where, Acontrol = absorbance of the control (ABTS+• solution without test sample) and
A sample = absorbance of the test sample (ABTS+• solution with test sample).

Calculation of 50 % inhibition concentration (IC_{50})

The IC50 values of ABTS radical cation were estimated. The comparison study was carried out using ascorbic acid and trolox as standards and test sample. The estimation was carried out in triplicate and the results were expressed as mean ± SD.

Lipid peroxidation inhibition Assay [38, 39]

Principle: The polyunsaturated fatty acid on cleavage produce malondialdehyde helps to estimate peroxide reaction. Malondialdehyde further combined with thiobarbituric acid (TBA) results in the formation of red colored thiobarbituric acid reacting substance (TBARS) observed at 532 nm.

Method:10 gm of rat liver tissue was weighed. The tissue was homogenized with a polytron homogenizer in phosphate buffer maintained at pH 7.4 results in the formation of 25% w/v homogenate. The homogenate was then centrifuged at 4000 rpm for 10 min. 0.1 ml of supernatant aliquot was combined with 0.1 ml of various samples in various concentration ranges from 5 to 100 µg/ml. Each of 0.1 ml of potassium chloride of 30mM, ascorbic acid of 0.06mM and ammonium ferrous sulphate of 0.16mM were added. The mixtures were incubated at 37ºC for 1 hr. After incubation the mixture was treated with 0.2 ml of Sodium dodecyl sulphate (8.1%), 1.5 ml of thiobarbituric acid (0.8 %) and 1.5 ml of 20 % acetic acid (pH 3.5). Made the volume upto 4 ml using distilled water.The mixture was kept in oil bath for 1 hr maintained at temperature 100ºC. After the cooling of mixture, 1ml of distilled water and 5 ml of 15:1v/v butanol-pyridine were added in the mixture. On vigorous shaking, mixture was centrifuged at 4000rpm for 10 min. The absorbance of the organic layer containing the thiobarbituric acid reactive substances (TBARS) was measured at 532 nm. A control was prepared using 0.1 ml of respective vehicle in the place of text compounds / BHT.

Calculation of 50 % inhibition concentration (IC50)

The optical density obtained with each concentration of selected test compounds and Butylated Hydroxyl Toluene (BHT). Percentage inhibition was calculated using equation 2. The IC50 value was determined by plotting graph taking concentration on X- axis and % inhibition on Y-axis.

$$\% \text{ Scavenging} = \frac{\text{Absorbance of control} - \text{Absorbance of test}}{\text{Absorbance of control}}$$

Xanthine oxidase (XO) assay [40]

Principle: In mammalian tissues, xanthine oxidase predominantly located in the liver and intestine. It catalyzes the hydroxylation of hypoxanthine to xanthine and then to uric acid and hydrogen peroxide. The activity was found to be low in liver injury and in blood. This leads to release of xanthine oxidase in the blood. The activity was up-regulated in cardiovascular and gout disorders. This colorimetric assay is based on XO-catalyzed oxidation of xanthine. The process where hydrogen peroxide was formed which was further catalyzed by peroxidase and reacts with 4-aminoantipyrine leads to the formation of product dye. The color intensity of the product is directly proportional to XO activity in the sample at 550nm.

Procedure: The xanthine oxidase activity measured spectrophotometrically at 295 nm. The mixture was prepared by mixing 50 mM of sodium phosphate buffer maintained at pH 7.5, sample solution dissolved in distilled water or DMSO, freshly prepared enzyme solution (0.2 U/ml of xanthine oxidase in phosphate buffer) and distilled water. The mixture was pre-incubated for 15 min at 37°C. The 0.15 mM of xanthine solution was added in the above prepared mixture. Further incubated mixture for 30 min at 37°C. 0.5 M hydrochloric acid was added to stop the further reaction. The absorbance was measured against a blank. Followed the same procedure for the preparation of blank sample, replaced the enzyme solution with the phosphate buffer. Prepared the control sample by using water/DMSO instead of test compounds in order to have maximum uric acid formation. Thus, xanthine oxidase activity was calculated using the following equation

$$\% \text{ XO inhibition } = \frac{1-\beta}{\alpha} \times 100$$

Where, α is the activity of XO without test sample and
β is the activity of XO with test sample

Superoxide radical scavenging assay [39]

Principle: In the assay superoxide (O2-), generated by light induced conversion of potassium superoxide to super oxide radical, converts to nitrobluetetrazolium to NBT-diformazan, which absorbs light at 560 nm. The reduced concentration of NBT-diformazan indicated reduced superoxide formation

Procedure: Superoxide scavenging was carried out using the alkaline Dimethyl sulphoxide (DMSO) method. Solid Potassium superoxide was allowed to stand in contact with dry DMSO for at least 24 hour and the solution was filtered immediately before use, filtrate (200 µl) was added to 2.8 ml of an aqueous solution containing Nitrobluetetrazolium (56 mol), EDTA (10 mmol) and Potassium phosphate buffer (10 mmol, pH 7.4). Test solutions at different concentrations (5-100 µg/ml) were added and then absorbance was recorded at 560 nm against the control in which pure DMSO had been added instead of alkaline DMSO. The percentage inhibition and 50 % inhibition concentration (IC50) was calculated

***In vitro* calcium oxalate assay**

The physicochemical theory of stone formation cogitates urine as a supersaturated solution in which homogenous or heterogeneous nucleation can lead to initiation of crystal formation, which can then aggregates and grow.

Nucleation Assay [41,2]

Solutions of calcium chloride and sodium oxalate were prepared separately at a final concentration of 3mM/L and 0.5mM/L respectively in a buffer containing Tris 0.5mM/L and NaCl 0.15mM/L of pH 6.5. Both the solutions were filtered thrice. For the assay, 950µl of calcium chloride and varying concentration of plant extract were pipetted out against a reagent blank (without extract). To this added 950µl of sodium oxalate and shook well at least for 2-3minutes. The absorbance was measured at 620nm.

Aggregation Assay

The CaOx crystal aggregation inhibition in presence of the extract of *T. procumbens* was determined by the method of [42] Hess et al. Calcium oxalate monohydrate crystal seeds were prepared by mixing calcium chloride and sodium oxalate at 50 mM/L. Both the solutions were equilibrated to 60°C in a water bath for 1hour and then cooled to 37°C overnight. The crystals were harvested by centrifugation and then evaporated at 37°C. Calcium oxalate monohydrate crystals were used at a final concentration of 0.8mg/ml buffered with Tris 0.05M / L and NaCl 0.15M / L at pH 6.5. Experiments were conducted at 37°C in the absence of the plant extract after arresting the stirring. The rate of aggregation (Ir) was estimated by comparing the slope of the turbidity in the presence of the extract with that obtained in the control.

$$Ir = \frac{\text{Turbidity of sample}}{\text{Turbidity of control}} \times 100$$

Growth assay

The percent inhibition of CaOx crystal growth that was induced in vitro was evaluated in the presence of *T. procumbens* extract by the procedure described by [43] Chaudary *et al*. 4mM calcium chloride and 4mM sodium oxalate of 1ml each were added to a 1.5ml of solution containing NaCl (10mM) buffered with Tris (10mM) at pH 7.2. To this 30µl of calcium oxalate monohydrate crystal slurry (1.5mg/ml acetate buffer) was added. Consumption of oxalate begins immediately after calcium oxalate monohydrate crystal slurry addition and was monitored for 600 seconds for the disappearance of absorbance at 214 nm. When plant extract was added into this solution, depletion of free oxalate ions will decrease if the extract inhibits calcium oxalate crystal growth. Rate of reduction of free oxalate was calculated using the baseline value and the value after 30 seconds incubation with or without the extract.

The relative inhibitory activity was calculated as follows:

$$\% \text{ relative inhibitory activity } = \frac{(C-S)}{C} \times 100$$

Where
C is the rate of reduction of free oxalate without any extract
S is the rate of reduction of free oxalate with *Tridax procumbens* extract.

RESULT AND DISCUSSION

Percentage yield of extract

Percentage yield of *Tridax procumbens* (leaves and stem) extract obtained in the various solvents presented in the table 1.

Table 1: Percentage yield of extracts

Sr. No.	Solvent	% Yield	
		Leaves	Stem
1.	Toluene	0.2%	0.3%
2.	Ether	0.15%	0.17%
3.	Ethanol	0.85%	0.97%
4.	Aqueous	0.94%	1.04%
5.	Flavonoid rich extract	0.49%	0.51%

Qualitative analysis of Phytochemical of *Tridax procumbens* leaves and stem extracts

The toluene and ether extract of leaves and stem of *Tridax procumbens* contains no or less amount of total phenol content. The total phenolic content in the ethanol extract of leaves and stem was found to be 1.27 % ± 0.42 and 1.31% ± 0.46 respectively. The aqueous extract of leaves and stem contains 1.85 % ± 0.71 and 1.97% ± 0.67 of phenol content and 0.96 % ± 0.22 and 1.09 % ± 0.39 phenolic content was found in the Flavonoid rich extract. There was no flavonoid content found in the leaves extracted in the solvent such as toluene and ether. The total flavonoid content in the ethanol, aqueous and flavonoid rich extract was found to be 0.82% ± 0.28, 1.08% ± 0.44 and 2.08 % ± 0.59 respectively. The amount of total flavonoid content was found to be no or less in toluene and ether extract of leaves and stem compared with the extracts in the solvent ethanol, aqueous and flavonoid rich extract. The study determined that the aqueous extract of *Tridax procumbens* show higher phenolic content whereas in flavonoid rich extract total flavonoid content was found to be more.

Table 2: Phytochemical analysis of *Tridax procumbens* leaves and stem extracts

Extracts	Leaves		Stem	
	Total Phenol Content	Total Flavonoids	Total Phenol Content	Total Flavonoids
Toluene	0.01% ± 0.12	--	0.17% ± 0.24	0.02% ± 0.35
Ether	--	--	0.04% ± 0.31	0.01% ± 0.29
Ethanol	1.27 % ± 0.42	0.82% ± 0.28	1.31% ± 0.46	0.76 % ± 0.72
Aqueous	1.85 % ± 0.71	1.08% ± 0.44	1.97% ± 0.67	1.14 % ± 0.85
Flavonoid rich extract (FRE)	0.96 % ± 0.22	2.08 % ± 0.59	1.09 % ± 0.39	2.19 % ± 0.69

Antioxidant study

The leaves and stem extracted in the various solvents were evaluated for DPPH, ABTS, lipid peroxidation and superoxide activity. The overall antioxidant action was found to be very good. The FRE of leaves (57.64± 0.44; 64.69± 0.69; 51.27± 1.06 and 49.28± 0.27) and stem (46.85± 1.64; 57.46± 1.28; 39.58± 0.29 and 46.75± 1.19) possess maximum DPPH, ABTS, lipid peroxidation and superoxide compared with the other extracts.

The leaves and stem of *Tridax procumbens* demonstrated a xanthine oxidase inhibitory activity extracted in different solvents given in the Table 3. The maximum inhibition was observed in the ethanol extract. The ethanolic extract of stem of *Tridax procumbens* showed higher inhibition (1724.25± 1.245) and ethanolic extract of leaves (1050± 0.12) and other extracts prepared from the same plant. Higher the inhibition of xanthine oxidase resulted in a decreased production of uric acid.

Table 3: IC50 in µg/ml for each activity

Extracts	DPPH		ABTS		Lipid peroxidation		Superoxide		Xanthine oxidase study	
	Leaves	Stem	Leaves	Stem	Leaves	Stem	Leaves	Stem	Leaves	Stem
Toluene	----	----	-----	----	----	----	----	----	----	----
Ether	----	----	-----	----	----	----	----	----	----	----
Ethanol	1679±0.33	1875±0.75	2465.0±0.84	2014.1±0.56	14752.2±1.26	1325±0.52	1958.64±0.22	1715.40±1.64	1050±0.12	1724.25±1.245
Aqueous	647.1±0.84	568.4±0.45	701±0.54	498.1±0.67	563.82±1.08	529.4±1.17	675.12±0.17	357.15±1.14	528.1±1.23	504.2±1.4
Flavonoid rich extract (FRE)	57.64±0.44	46.85±1.64	64.69± 0.69	57.46±1.28	51.27±1.06	39.58±0.29	49.28±0.27	46.75±1.19	48.25±1.02	38.46±1.58

Evaluation of effect of extracts for inhibition of calcium oxalate and brushite crystallization

Inhibition of CaOx crystal by Nucleation and aggregation assay:

The changes in the turbidity or optical density of different solutions, *viz* control, cystone (1000 µg/ml), and extracts (500 µg/ml and 1000 µg/ml), were plotted at different time intervals. The turbidity increased linearly up to 5 minutes, which indicated the nucleation process and then decreased linearly up to 15 minutes indicating the aggregation process. Extracts (500 µg/ml and 1000 µg/ml) and cystone (1000 µg/ml) inhibited both the rate of nucleation and the rate of aggregation. The percent inhibition rates of nucleation of CaOx by cystone (1000 µg/ml) and flavonoid rich extract of stem and leaves (1000 µg/ml) were found to be highest; 67.22, 57.73 and 49.64 percent, respectively. The percent inhibition rates of aggregation of CaOx by cystone (1000 µg/ml) and flavonoid rich extract of stem and leaves (1000 µg/ml) were found to be 75.94, 67.27 and 54.81 percent respectively. The photomicrographs of the CaOx crystals in solutions of control, cystone (1000 µg/ml) and flavonoid rich extract of stem and leaves (1000 µg/ml) showed that CaOx crystals were less dense in cystone (1000 µg/ml), flavonoid rich extract of stem and leaves (1000 µg/ml) as compared to control and other extracts.

Inhibition of CaOx crystal by growth assay

In calcium oxalate growth assay, Flavonoid rich extract (FRE) of stem and leaves inhibited calcium oxalate monohydrate (COM) growth. The percentage inhibition of cystone (1000 µg/ml), FRE of stem and leaves (1000 µg/ml) were 79.88, 62.43, and 71.61 respectively.

Table 4:Effect of *Tridax procumbens* extracts on percentage inhibition of crystal nucleation, growth and aggregation assays

Extracts	Nucleation		Aggregation		Growth	
	Leaves	Stem	Leaves	Stem	Leaves	Stem
Toluene	10.12	12.25	17.45	21.25	14.75	16.28
Ether	7.01	12.1	10.25	18.46	12.25	14.48
Ethanol	26.01	39.47	28.79	31.28	28.49	35.67
Aqueous	31.25	38.98	36.06	37.48	38.29	42.57
FRE	49.64	57.73	54.81	67.27	62.43	75.61
Cystone	67.22		75.94		79.88	

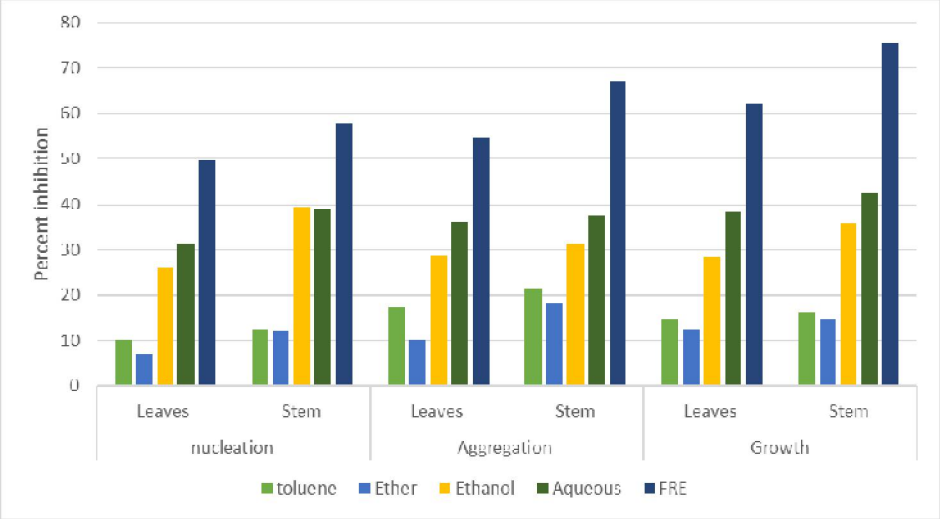

Figure 1: Effect of different extracts (1000 mircog/ml) concentraion on CaOx Nucleation, Aggregation and Growth

Inhibition of brushite crystal by Growth assay:

The growth of brushite crystals was measured as length (thickness of crystal deposition). The crystals acquired maximum length (approximately 0.35 cm) on day 3 after gelation took place and then after the length of the crystals deposited became constant up to day 8. The average length of the deposited CHPD crystals was decreased by citric acid (1 M), FRE of Stem and leaves (1000 µg/ml) until day 10. The length of the crystals in control, FRE of Stem and leaves (1000 µg/ml) were 0.41 cm and 0.19 cm, respectively; and in citric acid the crystals were too small to measure.

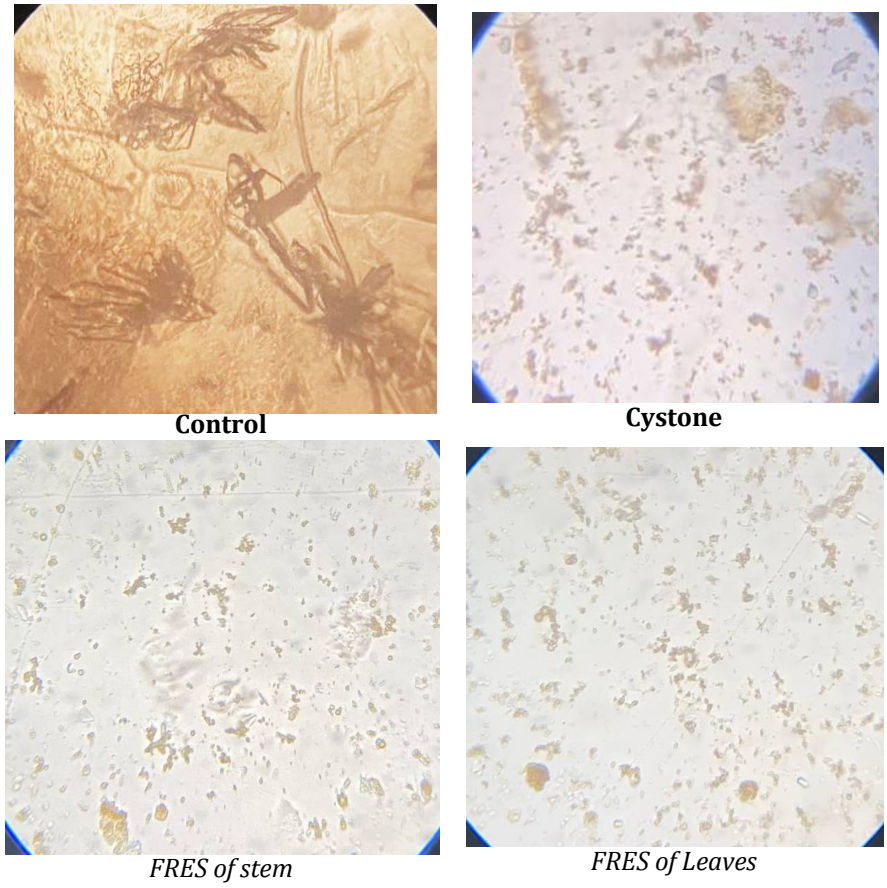

Figure 2: Photomicrographs of CaOx crystal density in different solutions

In vitro antiurolithiatic activity was conducted through solvent-solvent partitioning-based fractionation with the objective of achieving the concept regarding the category of phytoconstituents responsible for the litholytic efficacy of this herb. Nucleation refers to the formation of a solid crystal state in a solution and is the initial stage in crystal formation. The process of nucleation involves either homogenous nucleation or heterogenous nucleation. The former occurs in pure solution whereas, the latter takes place in divergent chemical environment such as the human urinary system and refers to epitaxial accumulation of crystals on pre-formed urinary crystals, cellular material or urinary casts. Stone formation commences with the urinary super saturation with stone forming components such as calcium, oxalate and phosphate leading to the formation of crystals, which serve as a nidus for crystal nucleation, growth, epitaxial growth and aggregation progressively leading to development of calculi. Therefore, a phytopharmaceutical agent capable of impeding the nucleation phase will also intervene with further stages of stone formation thereby preventing lithiasis. Restoration of the balance between urinary stone promoters (calcium, oxalate, uric acid, phosphate) and inhibitors (citrate and magnesium) needs to be attained by the intended antiurolithiatic agent as the imbalance contributes to lithiasis. Besides this, raising the urine volume, pH and anticalcifying activity, enhancing renal function and oxalate metabolism, lessening the oxidative stress on renal tissues are other mechanisms exhibited by medicinal herbs for alleviating the incidence of calculi.

CONCLUSION

The current research concluded that flavonoid rich extract revealed the greater inhibition on nucleation of calcium oxalate as compared to other extract-derived fractions of aerial parts and roots. Flavonoids and phenolics chiefly exhibit antioxidant activity and hence, can serve as antiurolithiatic agents by averting the crystal adhesion and consecutive formation of urinary stones by alleviating the injury caused by crystal accumulation in the urinary system that otherwise enhances the formation of free radicals resulting in destruction of membrane surface of kidney tubules via lipid peroxidation. Flavonoid rich extract from stem parts demonstrated an immense hindrance to nucleation which was as high as the standard formulation and the extract while toluene extract from leaves exhibited a lower activity than the standard and the other extracts.

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