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Antiurolithiatic activity of hydroalcoholic extract of *Wrightia tinctoria* R.Br. bark; an *in-vitro* study

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ABSTRACT
Urolithiasis has an important effect on the health care system. A large number of humans; nearly 10-15% of the human populations are suffering from urolithiasis (urinary or kidney stone) problem throughout the world with an approximate recurrence rate of 50% in 5-10 years and 75% in 20 years. The object of in-vitro antiurolithiatic study was to measure the inhibitory effect of hydroalcoholic extract of bark from W. tinctoria R.Br. on in-vitro crystallization, nucleation and aggregation of calcium oxalate as well as on crystal morphology in presence or absence of extract. The hydroalcoholic extract of bark from Wrightia tinctoria R.Br. was prepared by soxhletion and it was labeled as HAWT. The HAWT and Cystone were studied for their in-vitro antiurolithiatic activity. Time course measurements optical density at 620 nm were monitored by spectrophotometer in presence of HAWT at concentration (0.0625, 0.125, 0.250, 0.500, 0.750, 1.000 mg/mL) and standard drug Cystone (1.000 mg/mL) for inhibitory effect on in-vitro crystallization, nucleation, aggregation of calcium oxalate. It was observed that HAWT significantly inhibited the crystallization (86.35±0.08%), nucleation (75.60±0.04%) and aggregation (62.65±0.04%) of calcium oxalate and decreased the crystal density. Cystone was also inhibited the crystallization (94.42±0.08%), nucleation (78.04±0.04%) and aggregation (65.06±0.03%) of calcium oxalate significantly. The findings of present in-vitro antiurolithiatic study revealed that the HAWT (1.000 mg/mL) exhibited a very good inhibitory effect against experimentally induced urinary or kidney stone formation processes (crystallization, nucleation, and aggregation). It was concluded that phytotherapeutic agents from hydroalcoholic extract of W. tinctoria R.Br. bark have beneficial effect on urolithiasis and it may be useful as alternative or an adjunctive therapy in the management of urolithiasis.
Keywords: Urolithiasis, Urinary stones, Kidney stones, Cystone, *Wrightia tinctoria* R.Br.

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INTRODUCTION

The process of deposition or formation of stones in any part of the urinary system, including the kidney, ureters and urinary bladder is called Urolithiasis. Urolithiasis has an important effect on the health care system, a large number of people; nearly 10-15% of the human populations are suffering from urinary stone problem all over the world. Urolithiasis is more prevalent in men (12%) than in women (6%) and is more prevalent between the ages of 20-40 years in both sexes [1][2][3]. The urinary stone is an aggregation of crystals and solute materials from urine such as calcium, oxalate, phosphate, struvite, cystine and uric acid. Calcium oxalate is found to be the most predominant constituent of urinary stones. Calcium oxalate urinary stone represent up to 80% of analyzed stones. Calcium phosphate account for 15-25% and 10-15% is mixed stones, struvite 15-30%, cystine 6-10%, and uric acid stones 2-10%. Calcium oxalate stones are found in two different varieties, calcium oxalate monohydrate (COM) and calcium oxalate dihydrate (COD) [4]. The biochemical process involved in calcium oxalate stone formation is super-saturation, nucleation, crystal growth, aggregation, crystal retention, formation of stone. The interaction between crystal and renal tubular epithelial cell [5] including the adhesion [6] or endocytosis of crystal by cell and development of stone within the urinary tract. Currently, there are no satisfactory drugs in modern medicine, which can prevent or dissolve the stones and also reduce the recurrence rate of the urinary stone. Therefore, physicians remain to be depending on alternative systems of medicine for better relief. In India people living in different states utilize different plant parts and extract to treat or curing urolithiasis due to its safely, efficacy, cultural acceptability and lesser side effects.

The plant *Wrightia tinctoria* R.Br. (family- Apocynaceae) is the sweet variety of kutaja. *W. tinctoria* R.Br. is used as a traditional medicine for management of the dysentery, piles, psoriasis and other skin diseases. The extract from *W. tinctoria* R.Br. and their product has been extensively studied for its therapeutic

potentials and biological activities such as Antibacterial, Antidiabetic, Anti-psoriasis, Antidysenteric activity, Antimicrobial, Antifungal, Anti-inflammatory, Analgesic, Anthelmintics, Hepatoprotective and Wound healing activity. *W. tinctoria* R.Br. contain alpha-amyrin, beta-sitosterol, lupeol, ursolic acid and oleanolic acid [7]. *W. tinctoria* R.Br. needs to be explored for their pharmacological actions as antiurolithiatic agents.

MATERIAL AND METHODS

Plant material

Wrightia tinctoria R.Br. (Apocynaceae) bark was collected from the Gwalior, Madhya Pradesh (India) in August 2021. The plant specimen was identified from Govt. Autonomous Ayurvedic College, Gwalior, Madhya Pradesh (India) and authenticated from Botanical Survey of India, Central Regional Centre, Allahabad (India). A voucher specimen (S. No.- B.S.I./CRC/2020-2021/311) of the authenticated *W. tinctoria* has been deposited in the herbarium of the institute. The fresh bark was cleaned and separated from other extraneous matter and subjected to shade dried at room temperature. The dried bark subjected to a coarse powder in a mixer grinder and powder was stored in a non-toxic air tight plastic container at cool place.

Preparation of hydroalcoholic extract

The dried bark of *W. tinctoria* R.Br. powder (60 g) was extracted with hydro-alcohol (75% ethanol) using Soxhlet apparatus until the colorless extract obtained from extractor. The extract was concentrated on an electric water bath at 40-45°C to obtain brownish residue (Yield- 18.19 % w/w) a dry solid mass. It was labeled as HAWT and stored in a glass container for further studies. The HAWT were dissolved in distilled water to give concentrations of 0.0625-1.000 mg/mL and Cystone were also dissolved in distilled water to give concentrations 1.000 mg/mL.

Preliminary phytochemical screening of phytoconstituents

Preliminary phytochemical screening of hydroalcoholic extract of bark from *W. tinctoria* R.Br. was carried out to analyze the presence of various phytochemical constituents. The standard procedures described by Harborne [8], Evan [9] and Wallis [10] were used to identify the presence or absence of phytochemical constituents such as carbohydrate, flavonoids, alkaloids, tannins, phytosterols, glycosides, terpenoids, and saponins.

In-vitro antiurolithiatic activity

Crystallization assay

Crystallization assay was performed as previously described by Atmani *et al.* [11] and Hess *et al.* [12] with minor modifications. For crystallization assay, 5.0 mmol/L solution of calcium chloride and 5.0 mmol/L solution of sodium oxalate were prepared, in a buffer containing Tris 0.05 mol/L and NaCl 0.15 mol/L at pH 6.5. 950 μL Calcium chloride solution was transferred to eight tubes separately and allowed to maintained at 37 ^{o}C. 100 μL solutions of HAWT at different concentrations (0.0625, 0.125, 0.250, 0.500, 0.750, 1.000 mg/mL) and Cystone 1.000 mg/mL were added to each tube. Tubes with no extract added were used as control. Finally, 950 μL solution of sodium oxalate was added to each tube and these tubes were incubated at 37°C for 30 min. The optical density (OD) of solution was then recorded at 620 nm. The samples filtered through 0.25 mm membranes and the filtrate processed under the light microscope to analyses the density of formed crystals in the solutions. All crystallization assay were performed at least in triplicate.

Nucleation assay

The inhibitory activity of HAWT on the nucleation of calcium oxalate crystals was determined by the method of Atmani *et al.* [11], Hess *et al.* [12] and Wanjari *et al.* [13] with minor modifications. 5.0 mMol/L solution of calcium chloride and 5.0 mMol/L solution of sodium oxalate were prepared, in a buffer containing Tris 0.05 mol/L and NaCl 0.15 mol/L at pH 6.5. Both solutions were filtered three times through an 0.25 mm pore size filter paper. 950 μL Calcium chloride solution was mixed with 100 μL of the HAWT at various concentrations (0.0625, 0.125, 0.250, 0.500, 0.750 and 1.000 mg/mL) and Cystone 1.000 mg/mL into a 10 mm light path quartz cuvette placed in a spectrophotometer. Crystallization was started by adding of 950 μL sodium oxalate solution. The time course measurements optical density (OD) of the solution was monitored at 620 nm in spectrophotometer on every 10 sec over 5 min. The rate of nucleation was estimated by comparing the induction time (the delay before the appearance of crystals that have reached a critical size and thus become optically detectable) for calcium oxalate formation in the presence of HAWT at concentrations (0.0625, 0.125, 0.250, 0.500, 0.750 and 1.000 mg/mL) with that of the control (no extract). The percentage inhibition of nucleation was calculated by the following formula.

$$\text{Percent inhibition} = [(Tsc - Tsi)/Tsc)] \times 100$$

Where Tsc is the turbidity slope of the control (no extract), Tsi is the turbidity slope in presence of the inhibitors (HAWT and Cystone).

Aggregation assay

The inhibitory activity of the HAWT on the aggregation of calcium oxalate crystals was determined by the method of Atmani *et al.* [11], Hess *et al.* [14] and Saha *et al.* [15] with minor modifications. Calcium oxalate crystals were prepared by mixing 10 mmol/L calcium chloride and 10 mmol/L sodium oxalate. Both solutions were then equilibrated in a water bath for 1 hr. at 60 °C and then cooled to 37 °C overnight. The crystals were harvested by centrifugation and then evaporated at 37 °C. Calcium oxalate crystals were then dissolved with Tris 0.05 mol/L and NaCl 0.15 mol/L at pH 6.5 to a final concentration of 1.000 mg/mL. Experiments were conducted at 37°C and the absorbance at 620 nm was recorded at 30, 60, 90, 180 and 360 min. in the absence or presence of HAWT and Cystone. The rate of aggregation inhibition was estimated by comparing the slope of the turbidity in the presence of the HAWT and Cystone with that obtained in the control. The percentage inhibition was calculated as

$$\text{Percent inhibition} = [(Tsc - Tsi)/Tsc)] \times 100$$

Where Tsc is the turbidity slope of the control (no extract), Tsi is the turbidity slope in presence of the inhibitors (HAWT and Cystone).

Statistical analysis

All data were presented as mean ± SEM. Experimental data obtained were analyzed using one way ANOVA. P value less than 0.001 was considered statistically significant in all cases.

RESULT AND DISCUSSION

Preliminary Phytochemical Screening

Preliminary phytochemical screening of HAWT was carried out to analyze the presence of various phytochemical constituents. It was observed that the HAWT showed the presence of carbohydrate, tannins, alkaloid, flavonoids, sterols, and triterpenoid while glycosides, fixed oil, gum and resin were absent.

Effect of HAWT on crystallization

The *in-vitro* inhibitory effect of HAWT and Cystone on calcium oxalate crystallization was determined by the time course of turbidity measured at concentrations of 0.0625, 0.125, 0.250, 0.500, 0.750 and 1.000 mg/mL and Cystone (1.000 mg/mL) (Figure-1). The HAWT showed maximum percent inhibition of 86.35±0.08% at 1.000 mg/mL. On the other side, the Cystone showed maximum inhibition of 94.42 ± 0.08% at 1.000 mg/mL. The control (without extract) was found to show no inhibition of crystallization (Figure-2). Calcium oxalate crystals appear in different shapes, most notably as octahedral (envelope-shaped) or dumbbell-shaped crystals. These crystals are typically colorless and refract light, making them visible under light microscopy. The light microscopy in the control group showed the formation of both types of calcium oxalate crystals with significant aggregations (Figure-3a). HAWT at concentration (1.000 mg/mL) inhibited crystal formation less than Cystone (Figure-3b). Cystone was more effective than the HAWT with a smaller number of crystals when used at the same concentrations (Figure-3c). It was found that increasing the concentration of HAWT resulted in the increase in percentage inhibition of calcium oxalate crystallization. These results clearly demonstrated that HAWT has similar antiurolithiatic effects as compared to that in Cystone.

Effect of HAWT on nucleation

The *in-vitro* inhibitory effect of HAWT and Cystone on rate of nucleation was determined by the comparing the induction time course measurements of optical density (600 nm) at concentrations of 0.0625, 0.125, 0.250, 0.500, 0.750 and 1.000 mg/mL and Cystone (1.000 mg/mL). The HAWT showed inhibitory effect on nucleation significantly at various concentrations compared with Cystone (Figure-4). The HAWT showed maximum inhibition of 75.60±0.04% at 1.000 mg/mL, whereas Cystone showed maximum inhibition of 78.04±0.04% at 1.000 mg/mL (Figure-5). HAWT inhibited nucleation of calcium oxalate by disintegrating into smaller particles. These results clearly demonstrated that HAWT has similar antiurolithiatic effects as compared to that in Cystone.

Effect of HAWT on aggregation

The *in-vitro* inhibitory effect of HAWT and Cystone on rate of aggregation was determined by the comparing the slope time course measurements of optical density (600 nm) at concentrations of 0.0625, 0.125, 0.250, 0.500, 0.750 and 1.000 mg/mL and Cystone (1.000 mg/mL). The HAWT at various concentrations also had an inhibitory effect on aggregation, compared with Cystone. The HAWT showed maximum inhibition of 62.65±0.04% at 1.000 mg/mL and minimum of 13.25±0.04% was obtained at 0.0625 mg/mL, whereas Cystone showed maximum inhibition of 65.06±0.04% at 1.000 mg/mL (Figure-6). These results exhibit that HAWT has similar antiurolithiatic effects as compared to that in Cystone. It was found that as the concentration of HAWT increases, resulted in percentage inhibition of aggregation also increases.

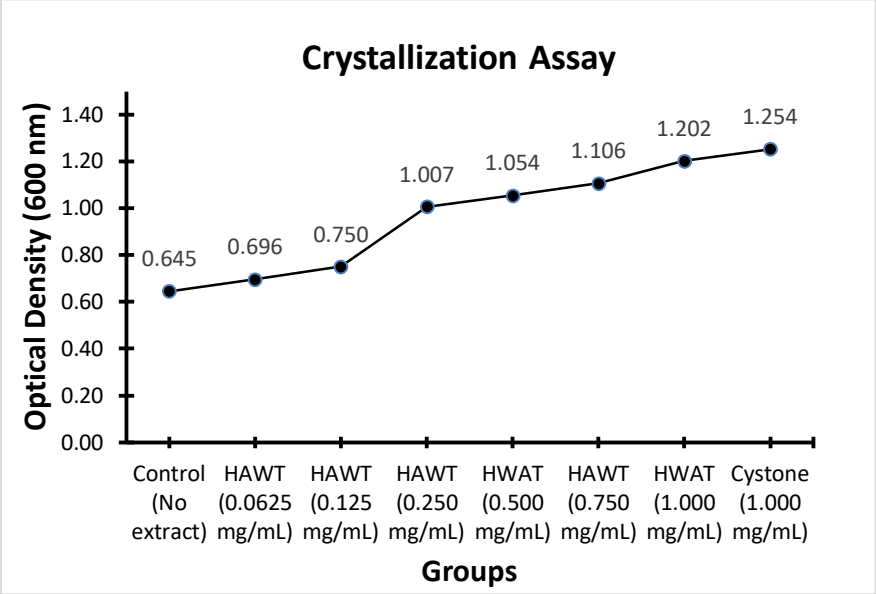

Figure-1: Optical density (620 nm) in presence of HAWT and cystone on crystallization

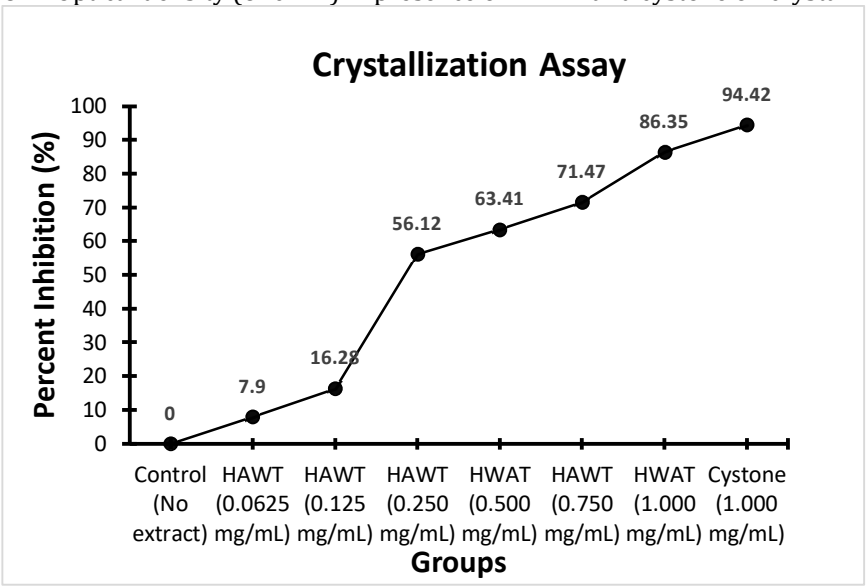

Figure-2: Percent inhibition effect of HAWT and Cystone on crystallization

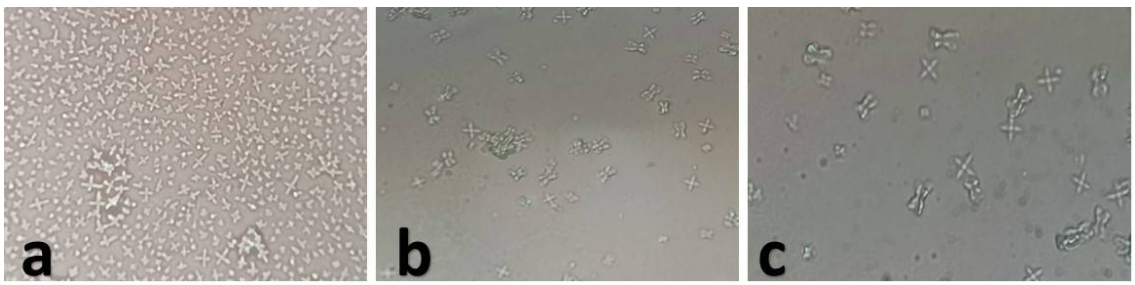

Figure-3: The induction of calcium oxalate crystallization in (a) control (no extract) (b) HAWT (1.000 mg/mL) and (c) Cystone (1.000 mg/mL) (light microscopy ×100)

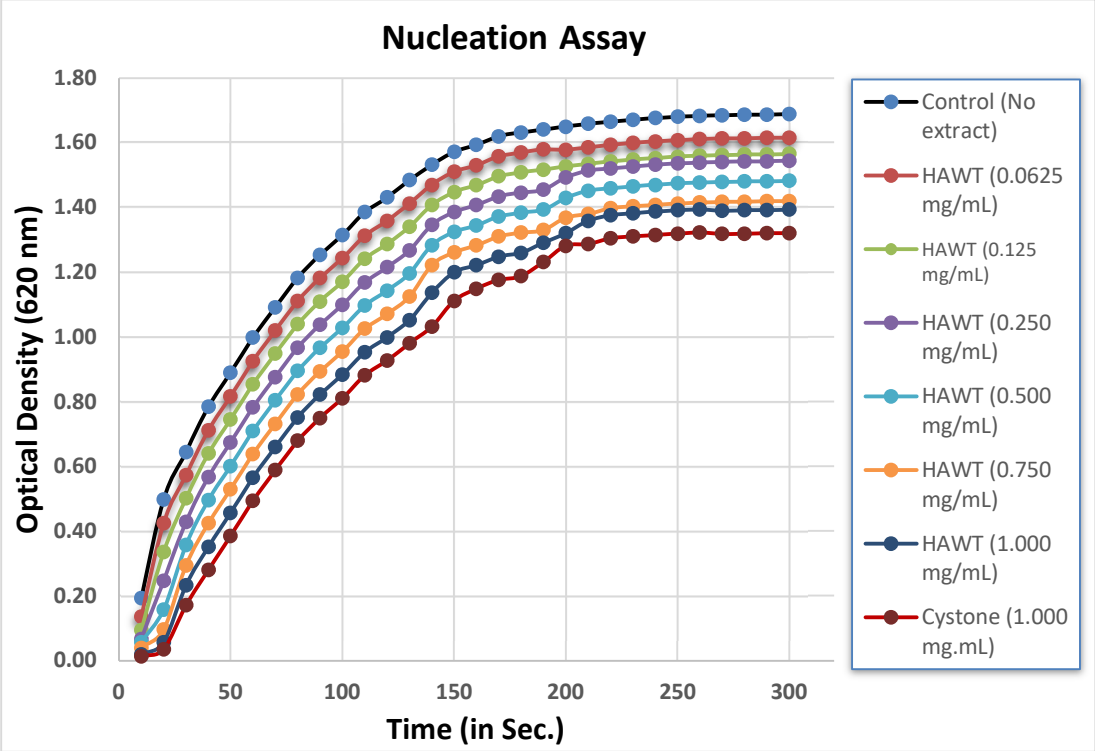

Figure-4: Time course measurements of optical density (620 nm) in presence of HAWT and cystone

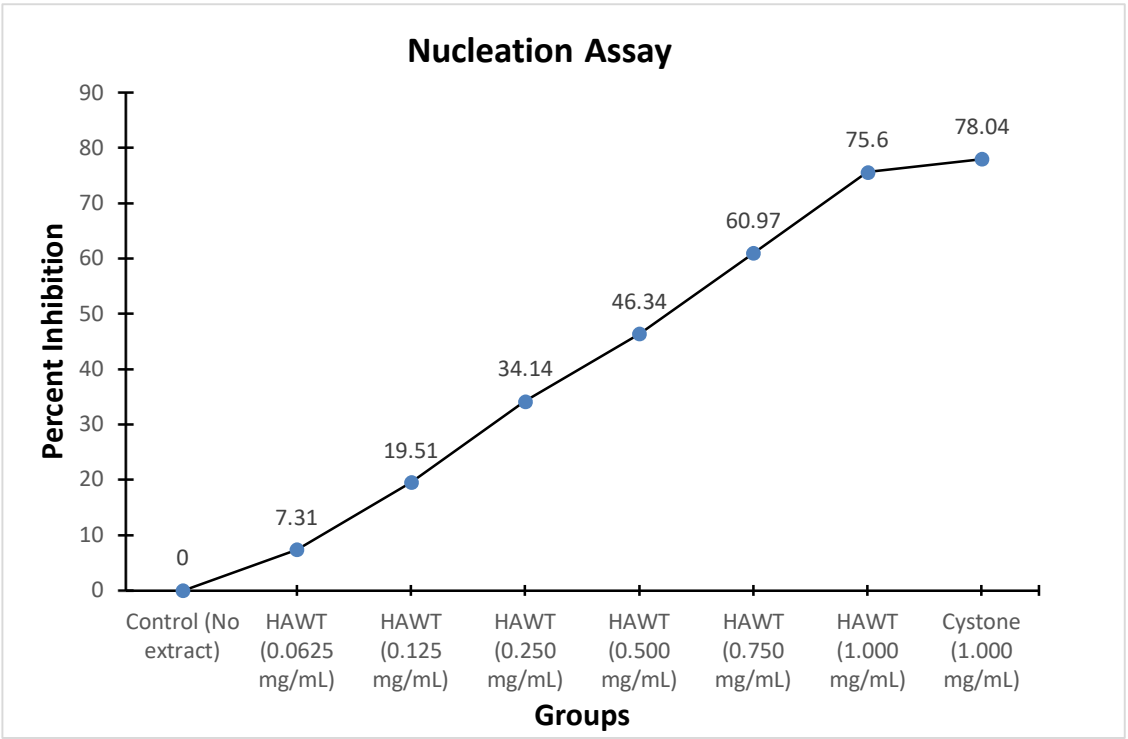

Figure-5: Percent inhibition effect of HAWT and Cystone on Nucleation. Values are the mean (±SEM) and were significantly different from the control (no extract), as well as within the groups, at P value less than 0.001

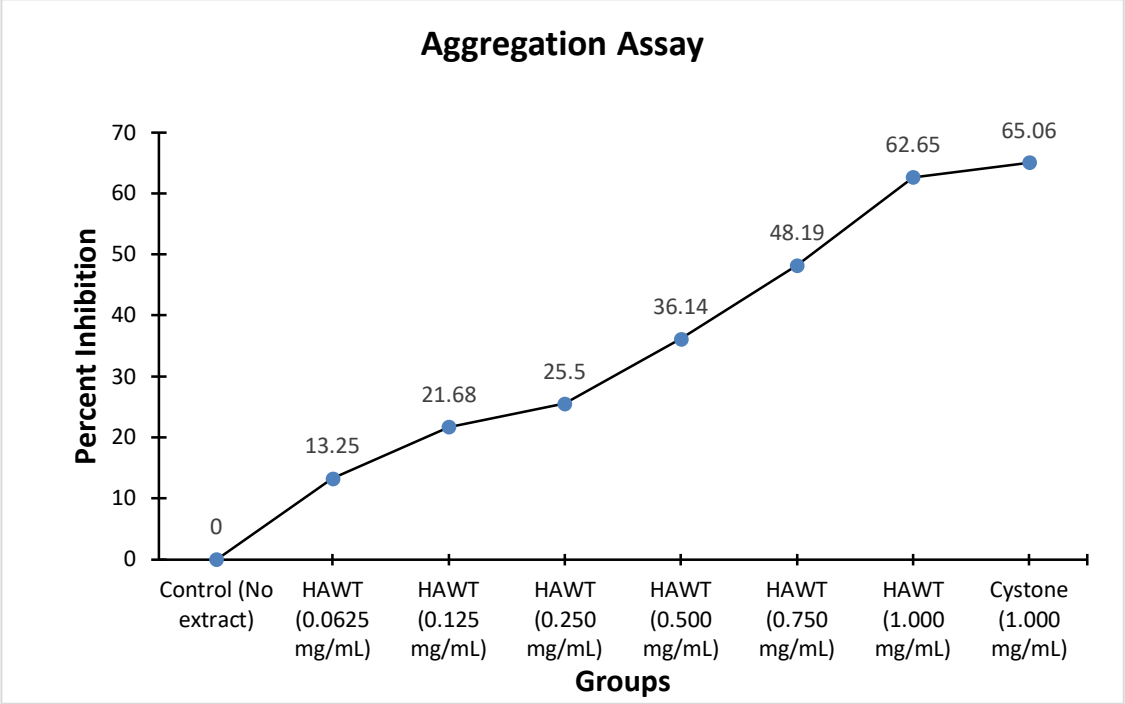

Figure-6: Percent inhibition effect of HAWT and Cystone on Aggregation. Values are the mean (±SEM) and were significantly different from the control (no extract), as well as within the groups, at P value less than 0.001

CONCLUSION

Urolithiasis is a process of formation or deposition of stones in any part of the urinary system, including the kidney, ureters and urinary bladder. Currently, there are no satisfactory drugs in modern medicine, having the property of disintegrating and dissolving the Urinary or kidney stone. This study evaluates the *in-vitro* antiurolithiatic activity of HAWT. The findings of the present *in-vitro* antiurolithiatic study revealed that the HAWT at 1.000 mg/mL concentrations exhibited a very good inhibitory effect against kidney stone formation processes (crystallization, nucleation, and aggregation). It was observed that HAWT significantly inhibited the crystallization (86.35±0.08%), nucleation (75.60±0.04%) and aggregation (62.65±0.04%) of calcium oxalate and decreased the crystal density. Cystone was also inhibited the crystallization (94.42±0.08%), nucleation (78.04±0.04%) and aggregation (65.06±0.03%) of calcium oxalate significantly. *In-vitro* nucleation and aggregation assay confirmed that HAWT contained nucleation inhibiting agents. HAWT is rich in triterpene [16], it may be responsible for antiurolithiatic activity. Furthermore, aforesaid activity was attributed to antilithiatic activity of a pentacyclic triterpene (lupeol) [17,18,19] thus, the triterpenoids present in the HAWT responsible for its preventive action against urinary or kidney stone formation. These phytotherapeutic agents may be useful as alternative or an adjunctive therapy in the management of urolithiasis. Further work will be continued to identify and isolate the potential phytotherapeutic agents for their pharmacological actions as antiurolithiatic agent.

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