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IN-VITRO AND *EX-VIVO* STUDIES ON SYNERGISTIC EFFECTS OF *LIMONIA ACIDISSIMA* AND APPLE CIDER VINEGAR ON ANTI-UROLITHIATIC ACTIVITY

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Keywords:

Anti-urolithiatic activity, Kidney stones, Synergistic studies, *Limonia acidissima*, Apple cider vinegar, Single gel diffusion, Degradation studies

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ABSTRACT: Kidney stones are aggregations of minerals such as calcium, oxalate, phosphates, uric acid, cysteine, *etc.* which can obstruct any part of the urinary system. This can turn fatal if left untreated as the major route of excretion is blocked causing the toxicity level to rise in the body which might lead to organ failure, coma and death. *Limonia acidissima* (wood apple) and Apple cider vinegar were found to contain phytoconstituents which are the bioactive components responsible for the anti-urolithiatic activity. Nucleation and aggregation of Calcium oxalate crystals was performed in presence of test samples were based on the percentage inhibition calculated, the mixture of samples gave the highest inhibition (37%) followed by wood apple (25%) and apple cider vinegar (9%). Calcium oxalate crystals were allowed to grow similarly where wood apple (113%) gave better inhibition when compared to apple cider vinegar (82%). The crystals obtained were microscopically monitored to correlate with obtained spectrophotometric results. Brushite crystals were grown synthetically by single gel diffusion and were exposed to the extracts. It was found that the mixture of samples synergistically inhibited the growth of crystals and also contributed towards the dissolution of the crystals. Surgically obtained kidney stones were subjected to degradation studies in the presence of the test extracts where the weight of stones was monitored periodically. The highest dissolution of the stones was observed in presence of mixture test extracts followed by wood apple and apple cider vinegar. The present study was successful in proving the synergistic effect of wood apple and apple cider vinegar towards the anti-urolithiatic activity.

INTRODUCTION: Kidney stones are formed due to supersaturation of various minerals like calcium, oxalates, phosphates, urates, cystine, *etc.* This may happen due to various reasons like tissue injury, lifestyle, urinary tract infections, genetic disorders, gout, *etc.* [1]

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Kidney stones can be majorly classified under five categories *i.e.*, calcium oxalate monohydrate / dihydrate stones, uric acid stones, struvite stones, cystine stones and drug-induced stones out of which calcium oxalate monohydrate stones are the most prevalent ones followed by calcium oxalate dihydrate stones, struvite stones, uric acid stones, cystine stones and drug-induced stones [2, 3].

Kidney stones can be fatal if it is left untreated as they block the normal flow of urine as they can get clogged anywhere in the urinary system. This can lead to various secondary infections all of which would finally lead to renal failure, shock, coma,

and death. Thus, the treatment of kidney stones is necessary to avoid these complications. Generally practiced methods of treatment are by using pharmacological drugs like thiazide diuretics, prednisolone, potassium citrate, *etc.* [4] Surgical intervention is required as the size of kidney stones cannot be dissolved by the regular drugs [5].

Limonia acidissima commonly known as wood apple is very rich in its phytochemicals like flavonoids, terpenoids, minerals like potassium, magnesium, etc. Ascorbic acid is one of the major components present in it which plays a key role in inhibiting the growth of kidney stones [6, 7]. This fruit has various activities such as antioxidant, wound healing, anti-diabetic, anti-hyperlipidaemic, anti-cancer, diuretic, hepatoprotective activity [8].

Apple cider vinegar is used in many home remedies to cure various small-scale ailments such as treatment for kidney stones. This might be due to the presence of various minerals like potassium, magnesium, many nutrients and a high amount of acetic acid [9, 10]. It has been found to contain various activities like antioxidant activity, antimicrobial activity, anti-diabetic activity, anti-obesity activity [11].

The present study was focused on investigating natural sources containing inhibitors of urolithiasis like terpenoids and to understand the urolithiatic activity by conducting nucleation, aggregation and dissolution experiments. Wood apple and Apple cider vinegar were the objects of study for investigating the anti-urolithiatic activity.

MATERIALS AND METHODS:

Samples and Sample Preparations:

Wood Apple: Unripe wood apple was purchased from the local market and it was ripened by the application of Calcium carbonate and exposure to the sun. The fruit was cracked open and the pulp along with seeds was sundried for 2 days. This was finely powdered using a grinder and stored for further usage. 10g of dried wood apple pulp in 100 mL of methanol was kept on a magnetic stirrer for 45 min and centrifuged at 8000 rpm for 15 min at room temperature and used freshly with required dilutions.

Apple Cider Vinegar: Puressenz Apple cider vinegar (with mother of vinegar) was bought from an organic supermarket and used accordingly with required dilutions. The dilutions were performed with filtered Millipore water.

Cystone (Standard Drug): Himalaya Cystone (Tablets 60) was obtained from a local pharmacy. 10g of peeled and crushed tablet in 100 mL of filtered Millipore water was kept on a magnetic stirrer for 30mins and centrifuged at 8000 rpm for 15 min, RT and used freshly with required dilutions.

Quantitative Estimation of Terpenoids: The terpenoid content of the samples was estimated according to the method proposed by Narayan Ghorai *et al.*, (2012). Sample preparations were conducted according to the methods prescribed previously. 200 µL of different sample supernatants were taken in micro centrifuge tubes. 1.5 mL of chloroform was added to all the tubes and vortexed. The solutions were incubated at room temperature for 3 min. 100 µL of concentrated sulphuric acid was added to all the tubes and vortexed. All the tubes were incubated in dark for 1.5 to 2 h. The supernatant was discarded and 1.5 mL of methanol was added to all the tubes to dissolve the precipitate. Absorbance was noted at 538 nm and the tests were conducted in triplicates [12].

Parameters to Test for Urolithiasis:

Nucleation and Aggregation Assay: Nucleation and Aggregation assay were performed as per the method previously described by Hess *et al.*, (2000) with minor modifications. Stock solutions of 10 mM $CaCl_2$ and 1 mM of Sodium oxalate solutions were prepared with a buffer solution containing 200 mM NaCl and 10 mM Sodium acetate (pH 5.7).15 mL of $CaCl_2$ solution was added into a clean beaker and maintained on a magnetic stirrer with continuous stirring at 37 °C. 1.5 mL of Control (Millipore water)/ Standard (10 mg/ mL cystone)/ 10 mg/ mL methanolic extract of wood apple/ 1:10 diluted ACV/ 1:1 mixture of wood apple and ACV was added under continuous stirring. Incubation time was started as soon as 15 mL of sodium oxalate solution was added. Absorbance was noted every minute at 620 nm for 30 min. All the crystallization experiments were conducted in triplicates. Percentage inhibition of the standard and extracts were calculated as

$$[1-(T_{si}/T_{sc})] \times 100$$

Where T_{sc} indicated the turbidity slope of the control and T_{si} indicates the turbidity slope in presence of inhibitor like cystone and extracts [7, 8].The effect of the test extracts on the crystals was monitored microscopically.

Calcium Oxalate Crystal Growth Assay: 10 mL of 4 mM Calcium chloride and 10 mL of 4 mM Sodium oxalate were added to 15 mL of Tris-NaCl (10 mM, pH 7.2) in a 50 mL beaker. 1 mL of distilled water (control) / Cystone-10 mg/ mL (standard) / (10 mg/ml) methanolic extract of WA/ 1:10 diluted ACV / 1:1 mixture of WA and ACV was added separately to the above solution. 300 µL of Calcium oxalate monohydrate (COM) crystal slurry (1.5 mg/ mL of acetate buffer pH-5.7) was added to the above reaction mixture (maintained on a magnetic stirrer at 450 rpm). Oxalate consumption by the COM added begins immediately which should be monitored for 10 min where the absorbance was recorded at 214 nm every 30 seconds. The rate of free oxalate consumption decreases if the test samples inhibit CaOx crystal aggregation. The relative inhibitory activity was calculated as [(C-S)/C] × 100 where C is the rate of reduction of free oxalate without any extract and S is the rate of reduction of free oxalate in the presence of test extracts [8, 9]. The effect of the test extracts on the crystals was monitored microscopically.

Single Gel Diffusion Growth Assay: Growth assay in single diffusion gel growth of brushite crystals was carried out according to the methods previously described by Joshi *et al.,* (2005) with some minor modifications. Calcium Hydrogen Phosphate Dihydrate (CHPD) crystals were grown until maximum growth was attained by single diffusion gel growth assay.

Glass test tubes of dimensions 2.5 cm in diameter and 15 cm in length were used to grow the crystals. 5 mL of Sodium metasilicate solution (specific gravity 1.06) was acidified with 1 M Ortho-phosphoric acid (approximately 1.4 mL) until pH-5.0 was obtained. The solution was quickly transferred into different test tubes and was allowed to rest until gelation occurred. 10 mL of 1 M aqueous solution of calcium chloride was added carefully on the set gel in all the test tubes. Crystals were found to be growing very rapidly within two days of incubation (at 37 ºC). Elongated platelet type crystals and star-shaped crystals were grown in the gel (observed in the microscope- 40x magnification). At the 8th day of the incubation period, all the test tubes were inoculated with 1 ml of citric acid/ 1% aqueous extract of cystone/ 1% methanolic extract of dried wood apple pulp/ 1:10 aqueous dilution of ACV and 1 mL of 1:1 mixture of WA and ACV extracts.

Control was maintained by inoculating the crystals in distilled water. The test was conducted in triplicates and all the test tubes were vortexed for even distribution of samples added. The size of the crystals was monitored every 2 days by morpho-logical examination at 10x magnification in a monocular microscope [10, 11, 12].

Kidney Stone Degradation Assay: Surgically removed human kidney stones were procured from M S Ramaiah Memorial Hospital, M S Ramaiah Nagar, Bengaluru, Karnataka, India. The assay was performed with reference to Rao and Bano's method, (2004) with some minor modifications. The length of kidney stones was measured (in cm) and the weight was recorded (in grams) and labelled. 40 mL of 0.05 M Tris-HCl buffer (pH-5.7) containing 0.15 M NaCl was dispensed in various sterile Tarson's containers and were labelled accordingly. 10 mL of 10% aqueous extract of cystone/ 10% methanolic extract of dried wood apple pulp/ undiluted apple cider vinegar and 1:1 mixture of WA and ACV extracts was added in respectively labelled containers.

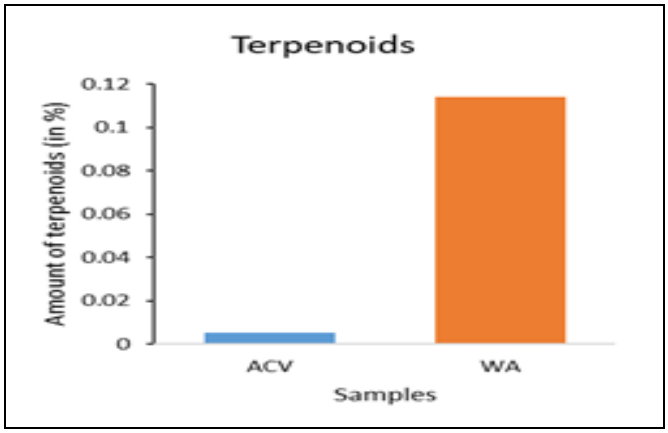
The evaluated and labelled kidney stones were inoculated in the respective containers. Control was maintained by inoculating the kidney stone in buffer containing 10 mL of distilled water. Few kidney stones were inoculated in a 1:1 mixture of ACV and WA in absence of buffer. The containers were vortexed daily to ensure equal distribution of the sample in the mixture.

The size and weight of the kidney stones were measured every 4 days by drying the washed stones at 100 ºC in a hot air oven for 5 min. The test was performed in triplicates for all the samples. % weight reduction was calculated as [13, 14]

% dissolution = [(Initial weight – Final weight) / Initial weight] × 100

RESULTS:

Quantitative Estimation of Terpenoids: The terpenoid content was estimated in 10% methanolic extract of dried wood apple pulp and 1:1 aqueous dilution of apple cider vinegar. It was found that WA contained a higher amount of terpenoids at 0.115 mg/ 100 mg when compared to ACV which contained 0.006 mg/ 100 mL of terpenoids. The results obtained were expressed as Linalool equivalents (LE).

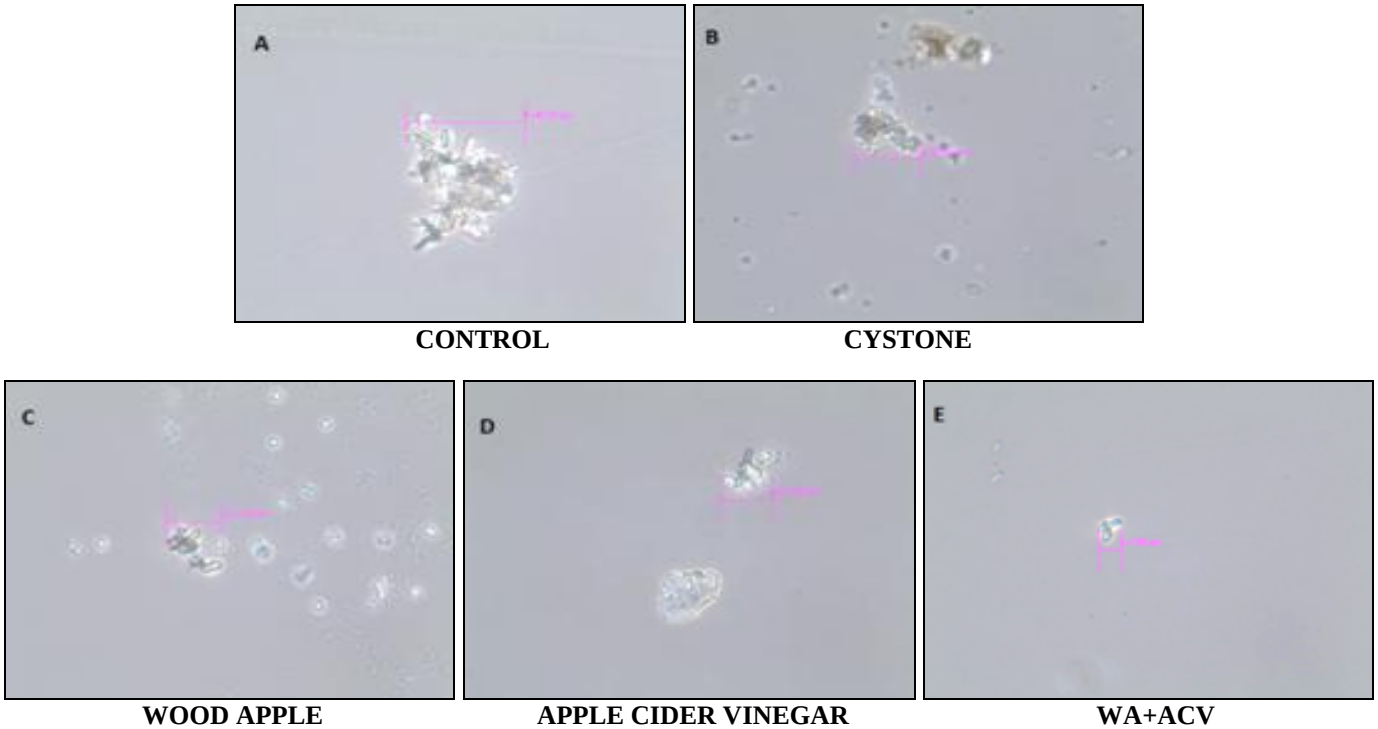


GRAPH 1: TERPENOID CONTENT IN WOOD APPLE AND APPLE CIDER VINEGAR (mg/100g OR mg/100mL) EXPRESSED AS MEAN

Nucleation and Aggregation Assay:

Microscopic Analysis: Kidney stone formation is initiated by forming small nuclear molecules of calcium oxalates which aggregate to form insoluble crystals/masses. In vitro studies of 10 mg/mL methanolic extract of Wood apple showed promising results in inhibition of nucleation and aggregation of calcium oxalate crystals. Various phytoconstituents estimated in the samples like flavonoids, terpenoids, organic acids, antioxidants and various minerals like K^+ and Mg^{2+} possess anti-urolithiatic activity thus contributing towards inhibition of nucleation and aggregation of CaOx nuclei.

Aggregation is an important factor in the genesis of stones. 1:10 diluted ACV showed betterinhibition of nucleation than the standard drug cystone and 10% methanolic extract of WA. The mixture of 1:10 diluted ACV and 10 mg/ mL methanolic extract of Wood apple (in a ratio of 1:1) showed the highest inhibition of nucleationwhen compared to all the other samples. Based on the conclusions derived from microscopic evaluation, the samples inhibit nucleation synergistically.



CONTROL **CYSTONE**

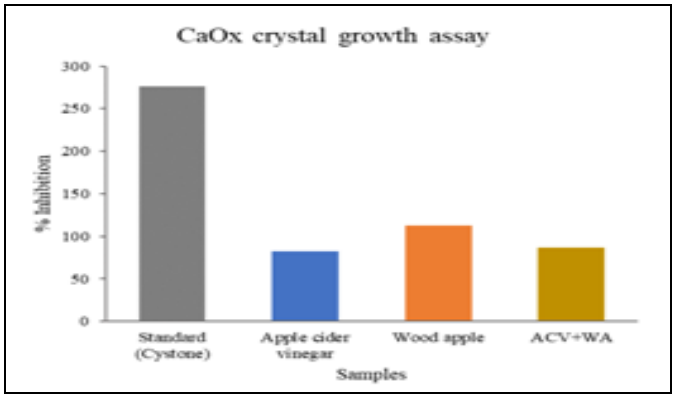
WOOD APPLE **APPLE CIDER VINEGAR** **WA+ACV**

FIG. 1: PHASE CONTRAST MICROSCOPY AT 40X MAGNIFICATION IN THE ABSENCE AND PRESENCE OF TEST EXTRACTS. (NUCLEATION AND AGGREGATION ASSAY)

Calcium Oxalate Crystal Growth Assay: In CaOx crystal growth assay the rate of Calcium oxalate monohydrate crystal growth is monitored in the presence and absence of WA and ACV. Free oxalate ions aggregate on COM crystals contributing to the increase in the size of the

crystals. The rate of aggregation can be monitored at 214 nm by checking for the decrease in free oxalate ions in the solution causing a reduction in absorbance.

Spectrophotometric Analysis:

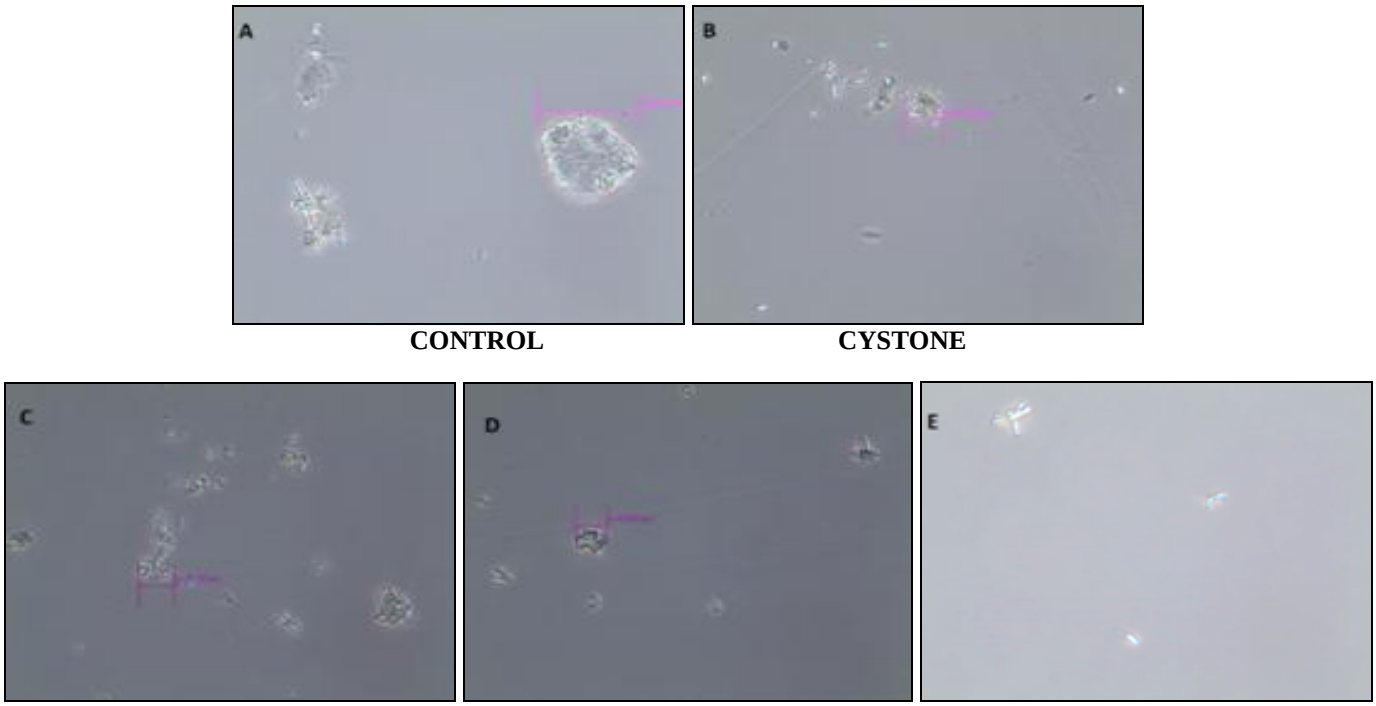


GRAPH 2: PERCENTAGE INHIBITION OF CALCIUM OXALATE CRYSTAL GROWTH IN THE PRESENCE OF SELECTED SAMPLE EXTRACTS EXPRESSED AS MEAN

Microscopic Analysis: According to the results obtained1% cystone extract showed the highest % inhibition of aggregation at 276.6635711 followed by 1% methanolic extract of Wood apple at 112.5917055. The lowest of all the test samples was found to be 1:10 aqueous dilution of ACV at 82.32175357%.

The mixture of WA and ACV at a 1:1 ratio showed % inhibition of CaOx crystal growth greater than % inhibition of ACV and lesser than % inhibition of WA.

It can be inferred from this obtained result that there is a slight antagonism between WA and ACV extracts in terms of inhibition of CaOx crystal growth. The results can be correlated by observing the morphological changes of the crystals in the presence of test extracts.



CONTROL **CYSTONE**

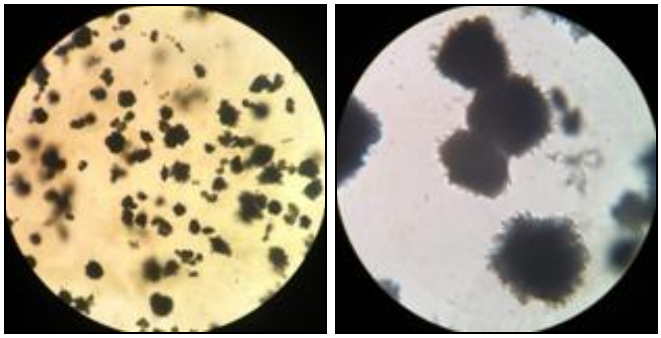
WOOD APPLE **APPLE CIDER VINEGAR** **WA+ACV**

FIG. 2: PHASE CONTRAST MICROSCOPY AT 40X MAGNIFICATION IN THE ABSENCE AND PRESENCE OF TEST EXTRACTS.(CAOX CRYSTAL GROWTH ASSAY)

Single Gel Diffusion Growth Assay: Sodium metasilicate gel was used to grow calcium hydrogen phosphate dihydrate (CHPD) crystals where the diffusing compound was calcium chloride. The size of the crystals was monitored by observing under 10x magnification in a monocular microscope at an interval of 2 days. Final observations were conducted at the end of the 10th day starting from the day of inoculation with samples after maximum growth of crystals was achieved. According to the results obtained, 1M citric acid-containing gel showed complete dissolution of CHPD crystals. The effect of citric acid corroborates with previous finding 15. 1:1 mixture of WA and ACV showed significant inhibition of growth of brushite (CHPD) crystals

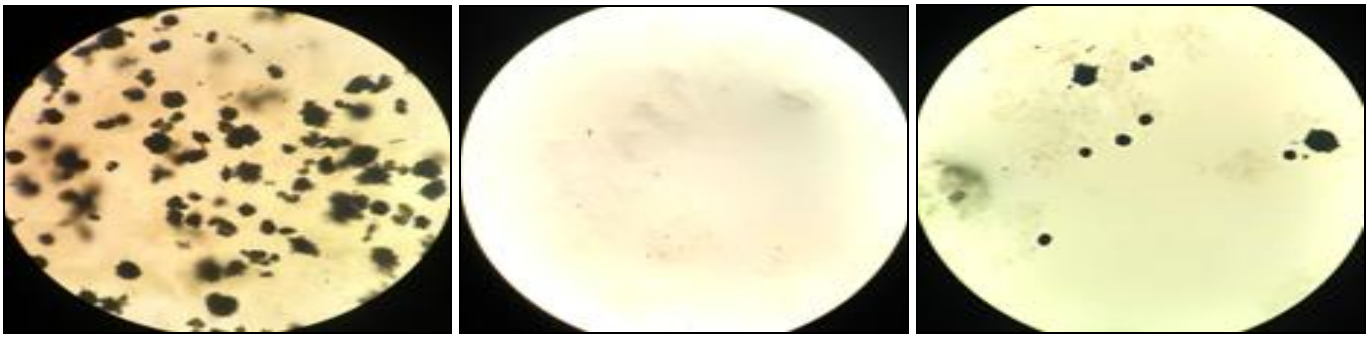
and caused their dissolution as observed under 10x magnification of a monocular microscope when compared to all other test samples. According to the observations, it can be concluded that ACV has better growth inhibition properties when compared to cystone and WA.

This can be concluded based on the number of CHPD crystals seen under the microscopic field. WA and cystone have better dissolution capacity as the size of crystals seen under the microscopic field are lesser than ACV.

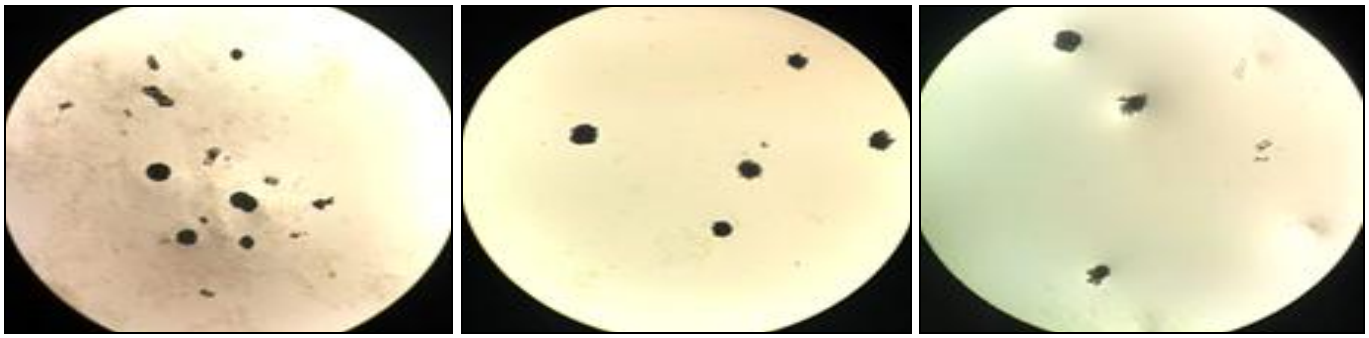


10X MAGNIFICATION **40X MAGNIFICATION**

FIG. 3: CHPD CRYSTALS ON DAY 0 OF INOCULATION (8TH DAY OF CRYSTAL GROWTH)



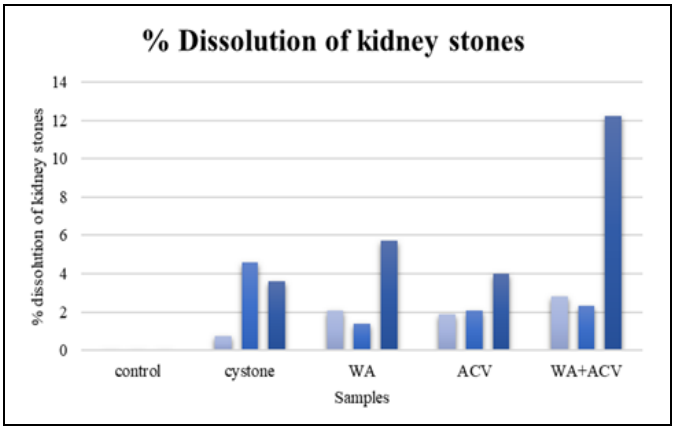
CONTROL **CITRIC ACID** **1% CYSTONE**



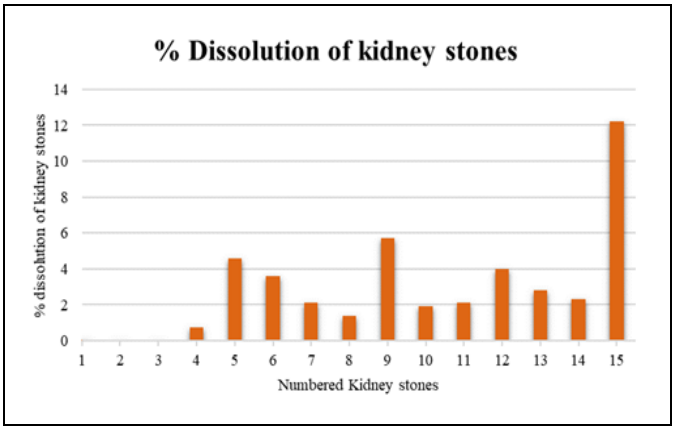
WOOD APPLE **APPLE CIDER VINEGAR** **WA+ACV**

FIG. 4: MICROSCOPIC IMAGES OF CHPD CRYSTALS ON 10TH DAY OF INOCULATION WITH VARIOUS SAMPLES

Kidney Stone Degradation Assay: Surgically removed human kidney stones can be of four kinds which were mentioned previously. This can be analyzed by various analytical methods like Fourier-transform infrared spectroscopy (FTIR). The present study was conducted to analyse the dissolution capacity of the selected samples on the previously weighed kidney stones. Previously weighed kidney stones were incubated in the sample of choice along with buffer (pH-5.7) at 37 ℃. The weight of kidney stones was monitored for 24 days with an interval of four days. Based on the results obtained, the mixture of WA and ACV (1:1) gave the highest percentage dissolution of kidney stones on the 24th day. This was followed by a 10% methanolic extract of dried WA pulp followed by standard drug cystone and finally, ACV gave the least amount of dissolution of kidney stones.



GRAPH 3: % DISSOLUTION OF KIDNEY STONES BASED ON EXPOSURE TO VARIOUS SAMPLES



GRAPH 4: % DISSOLUTION OF KIDNEY STONES BASED ON THE SIZE OF STONES

When the initial weight and final weight of the kidney stones were compared, it was found that the initial weight of the stones determined the rate of dissolution. For instance, stone number 7 having an initial weight of 0.3521 g showed 2.1% dissolution of the stone under exposure to WA extract but another stone 9 exposed to the same gave 5.7% dissolution as the initial weight of the stone was 0.078 g. Hence it can be concluded that the size of the stone determines the rate of dissolution of the stone *i.e.,* smaller the stone, greater the dissolution, and vice versa.

CONCLUSION: The present study was successful in understanding the effect of the chosen samples on kinetics and dynamics of kidney stones. The effect of samples was studied on the formation of kidney stones and dissolution capacity was also calculated. Phytoconstituents of wood apple (*Limonia acidissima*) and Apple cider vinegar was estimated to understand the relation between the concentration of phytoconstituents and the effect on formation and degradation of kidney stones. Wood apple was found to contain high amount of terpenoids (0.114 mg / 100g dry weight) where as Apple cider vinegar contained very minute amount of terpenoids (0.005 mg / 100 mL).

Wood apple pulp extracts gave 25% inhibition for nucleation and aggregation assay and 11.3% inhibition for calcium oxalate crystal growth assay whereas apple cider vinegar showed 9% inhibition of nucleation and aggregation and 8.2% inhibition of calcium oxalate crystal growth. By comparing the obtained results, it can be concluded that wood apple pulp has better inhibition activity when compared to apple cider vinegar.

Microscopic examination of crystals in presence of test extracts revealed that wood apple extracts have significant activity in prevention of aggregation of crystals and crystal growth. On the other hand, under dissolution studies on CHPD crystals and surgically obtained kidney stones it was found that apple cider vinegar showed better dissolution of the stones and crystals when compared to wood apple.

Further studies were conducted for all the parameters with the combination of test extracts to understand the synergism and antagonism between the samples. Synergism was seen significantly in between the extracts in nucleation and aggregation assay where the mixture of test extracts showed highest inhibition of nucleation and aggregation of calcium oxalate crystals at 37%. Antagonism was observed slightly in between the test extracts in calcium oxalate crystal growth assay *i.e.,* 8.7% of inhibition whereas wood apple showed highest value at 11.3% and apple cider vinegar gave 8.2% of inhibition of growth of CaOx crystals.

Synergism was also seen between the test extracts in terms of inhibition of growth of CHPD crystals and dissolution of CHPD crystals. Effect of test extracts on biologically obtained kidney stones was also evaluated by incubating the stones in test extracts in presence of buffer. It was noted that mixture of samples gave the highest dissolution of kidney stones followed by wood apple extracts and apple cider vinegar. The dissolution of stones was also found to be related to the initial weight of the stones. Smaller the stone size, greater was the dissolution of these stones. Hence the dissolution capacity was evaluated based on the initial weight of the stones and the sample acting upon them. The percentage dissolution of stone number 15 was the highest out of all the stones at 12.2% as the initial weight of the stone was low and the effective sample was the mixture of wood and apple cider vinegar extracts. Finally, the conclusion of the study can be drawn as combination of test extracts has better anti-urolithiatic activity. Wood apple and apple cider vinegar equally contribute in inhibition of urolithiasis where each of these extracts contribute in a unique way.

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CONFLICTS OF INTEREST: There was no conflict of interest during the research work.

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