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In Vitro Studies on Synergistic Effects of Limonia Acidissima And Apple Cider Vinegar on AntiUrolithiatic Activity

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Abstract

Traditionally Wood apple (*Limonia acidissima*) and Apple cider vinegar was found to be used to treat and prevent kidney stones. The present study is dedicated towards proving the same and providing scientific proof to these traditional ideas. Minerals like calcium, oxalates, phosphates, uric acid etc. form insoluble aggregates through formation of monohydrates and di-hydrates. This process is initiated by nucleation and then aggregation of insoluble masses. Phytoconstituents like flavonoids, terpenoids, ascorbic acid, acetic acid, potassium, magnesium etc. contain inhibitory properties and hence are urolithiatic in nature. In vitro tests for nucleation and aggregation has been conducted for Wood apple, Apple cider vinegar and their (1:1) synergistic sample and absorbance at 620 nm was recorded to obtain a turbidity slope. The tests were conducted in presence of sodium acetate/ NaCl buffer of pH 5.7 to mimic the biological conditions. 1:10 dilution of commercially obtained Apple cider vinegar sample showed better % inhibition of nucleation and aggregation when compared to the inhibition shown by standard drug cystone. 10 mg/ mL methanolic extract of dried pulp of wood apple showed higher % inhibition when compared to cystone and Apple cider vinegar. Synergistic sample containing both wood apple and apple cider vinegar in equal proportion showed the highest % inhibition for nucleation and aggregation of calcium oxalate crystals. This proved the synergistic activity of wood apple and apple cider vinegar for urolithiasis. Further studies are being conducted check for aggregation and degradation activities of the mentioned test extracts.

Keywords

Anti-urolithiatic activity, Apple Cider Vinegar, Wood Apple, Synergistic effect, Nucleation and Aggregation.

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1. INTRODUCTION:

Kidney stones are hard masses of crystals formed due to aggregation of dissolved minerals in the kidneys or urinary tract or the bladder. These are also known as renal calculus. Commonly the minerals responsible for the formation of theses masses are calcium, oxalates,

phosphates, uric acid, cystine and other similar minerals. Medically kidney stones are categorised into four different types of which the most prevalent form is calcium oxalate monohydrate/dihydrate stones followed by struvite stones, uric acid stones and the least common kind is cystine stones. Kidney stones can be treated through invasive keyhole surgeries or noninvasive methods like oral medications or laser treatment. The urinary calculi (= urinary stones, renal stones or kidney stones) may arise due to chemical activities within the body [1]. The condition favorable for the formation of the stones includes high concentrations of calcium salts (Ca-oxalate and Ca-phosphate), infections of urinary tracts, pH, and a decrease in the body's natural ability to inhibit the formation of the crystals [1].

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. This fruit has various activities such as antioxidant, wound healing, anti-diabetic, anti-hyperlipidemic, anti-cancer, diuretic, hepato-protective activity [2].

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 high amount of acetic acid. It has been found to contain various activities like antioxidant activity, antimicrobial activity [3], anti-diabetic activity, anti-obesity activity[4].

The present study is dedicated towards identifying the potential of anti-urolithiatic activity of the fruit pulp of *Limonia acidissima* (wood apple) and Apple Cider vinegar (ACV). Along with this we are checking for the synergistic/antagonistic effect of the combined samples on urolithiasis.

2. MATERIALS AND METHODS:

Wood apple was procured from the local market in Bengaluru and was ripened. The extracted pulp was sun dried along with the seeds, powdered and stored in airtight container. The Apple Cider Vinegar was bought at a local organic store and used in various dilutions. The chemicals and solvents required were provided by the laboratory, Department of Biochemistry, Mount Carmel College, Bangalore, India.

2.1. Phytochemical screening: Phytochemical evaluation of dried extracts of wood apple was conducted with methanol and water to check the solubility and activity. 10% extract of methanol and water was prepared by placing the mixtures on magnetic stirrer for 45 mins and centrifuged at 8000 rpm, 15 mins, 24°C. The obtained clear supernatants was subjected to various phytochemical tests. ACV was diluted with distilled water and filtered. The different dilutions tested were 1:5, 1:10 and 1:20. These dilutions were also subjected to various phytochemical tests like carbohydrates, proteins, alkaloids, glycosides, tannins/phenolics, flavonoids and terpenoids [5,6,7,8].

2.2. Quantitative estimation of Flavonoids: 1g of dried sample was macerated with 10 mL of (8:2) methanol: water and filtered with whatman filter paper. The filtrate was partially dried and 6 drops of 2M H_2SO_4 was added. Chloroform was added to acidified filtrate in 3:1 ratio in separating funnel. The mixture was shaken well for 10 mins and chloroform layer was extracted from the separating funnel after 30 mins which was dried for 12 hours. The dried extract was dissolved in minimal amount of methanol for further tests [9].

0.2 to 1.0 mL (10µg/mL) aliquots of standard quercetin solution was pipetted out into different test tubes which was made upto 2.0 mL with methanol. 2.0 mL of methanol was taken as blank and 1.0 mL of extracts were taken separately for flavonoids estimation. 0.1 mL of 10% $AlCl_3$ and 0.1 mL of 1M potassium acetate solutions were added to all the test tubes which were incubated for 30 minutes at room temperature. The absorbance was checked at 415 nm for all the test tubes and standard graph was plotted to estimate the flavonoid content of extracts as quercetin equivalents. The test was conducted in triplicates [10].

2.3. Quantitative estimation of Ascorbic acid in Wood apple: 10% methanolic extract of dried pulp of wood apple was used for Ascorbic acid estimation. Standard solutions of 2,6 - dichlorophenol indophenol and 1% ascorbic

acid was prepared with oxalic acid-acetic acid activation mixture. The DCPIP solution was standardized by titrating with 1 mL of standard ascorbic acid solution mixed with 10 mL of activation mixture. This standardized DCPIP solution was used to titrate with 1 mL of 10% methanolic wood apple extract mixed with 10 mL of activation mixture. The end point was pale pink. All the tests were conducted in triplicates [11].

2.4. Quantitative estimation of Acetic acid in Apple Cider Vinegar: 1:1 dilution of ACV with distilled water was prepared and filtered and used for acetic acid estimation. Standard solutions of sodium hydroxide and oxalic acid were prepared with distilled water. NaOH was standardized with 10 mL of 0.05M of oxalic acid. 0.5% phenolphthalein was used as indicator which turned the solution to pale pink in presence of base which was considered as the end point. 25 mL of extract was titrated against standardized NaOH in presence of indicator until end point was obtained. All the tests were conducted in triplicates [12].

2.5. Parameters to test for Urolithiasis

2.5.1. Nucleation and Aggregation assay: Nucleation and Aggregation assay were performed as per method previously described by Hess et al. [2000] with minor modifications [13]. Stock solutions of 10 mM $CaCl_2$ and 1 mM of Sodium oxalate solutions were prepared with buffer solution containing 200 mM NaCl and 10 mM Sodium acetate (pH 5.7). All the solutions were prepared with filtered Millipore water and again filtered after preparations. 15 mL of $CaCl_2$ solution was added into a clean beaker and maintained on 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 minutes [14]. The blank was set with millipore water. All the crystallization experiments were conducted in triplicates. Percentage inhibition of the standard and extracts were calculated as [1-(Tsi/Tsc)] ×100 where Tsc indicated the turbidity slope of the control and Tsi indicates the turbidity slope in presence of inhibitor like cystone and extracts [15,16].

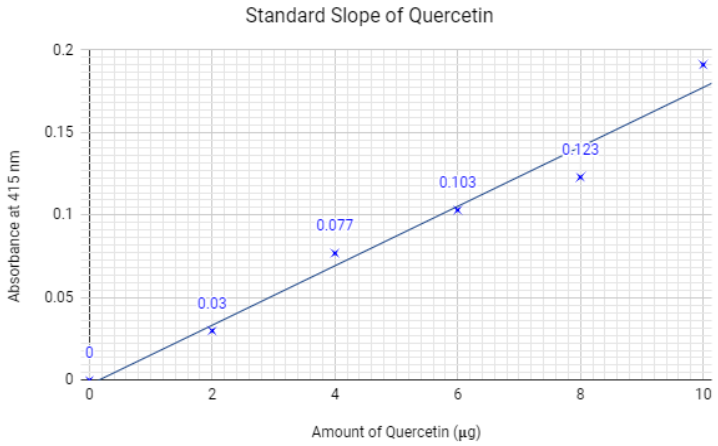
3.OBSERVATIONS AND RESULTS:

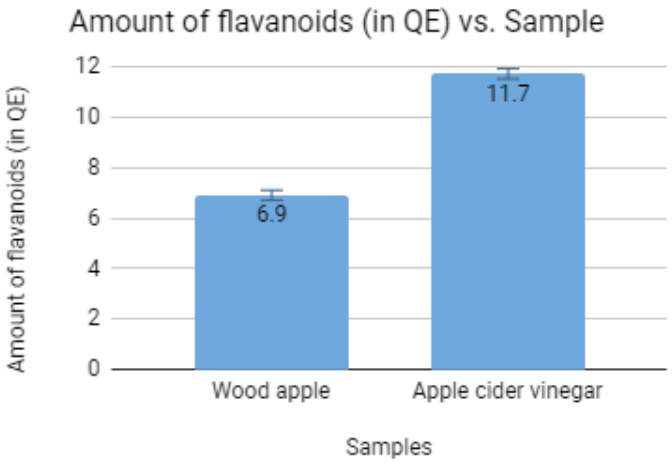
3.1. Preliminary phytochemical screening:

	Wood Apple - Dried Pulp 10% w/v Extract		Apple Cider Vinegar - Dilutions with Water		
Test	**Water**	**Methanol**	**1:5**	**1:10**	**1:20**
Molish	++	+++	+++	++	++
Fehlings	+++	+++	+++	++	++
Proteins	-	+	+	-	-
Alkaloids	+	+	-	-	-
Glycosides	+	++	++	+	-
Tannins/Phenolics	-	+	-	-	-
Flavonoids	+++	+++	+++	++	+
Terpenoids	++	+++	+++	++	+

From the above table it was found that methanolic extract of wood apple gives better results when compared to the aqueous extracts. Hence methanolic extract was taken for further analysis. In case of ACV, better results were found in 1:10 dilution and hence this dilution was considered for further analysis. It was found that wood apple and ACV were rich in flavonoids and terpenoids and they were subjected to quantitative estimations.

3.2. Quantitative estimation of Flavonoids:





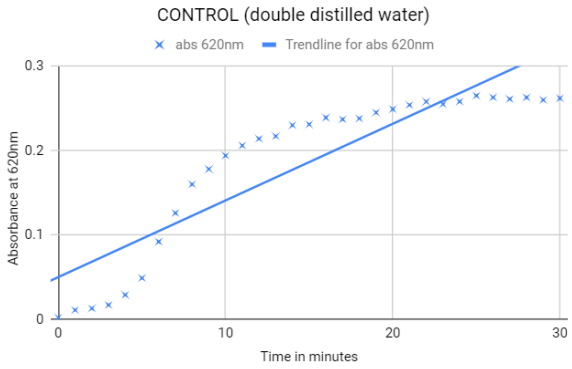
Wood apple was found to contain 6.9 ± 0.15 µg QE/g sample dry weight of flavonoids. Similarly, ACV was found to contain 11.7 ± 0.3 µg QE/ mL sample of flavonoids.

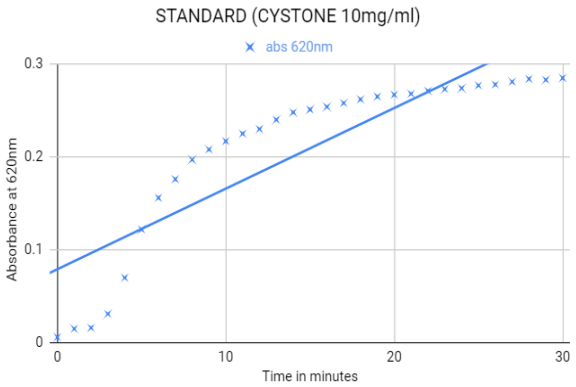
3.3. Quantitative estimation of Acetic acid and Ascorbic acid

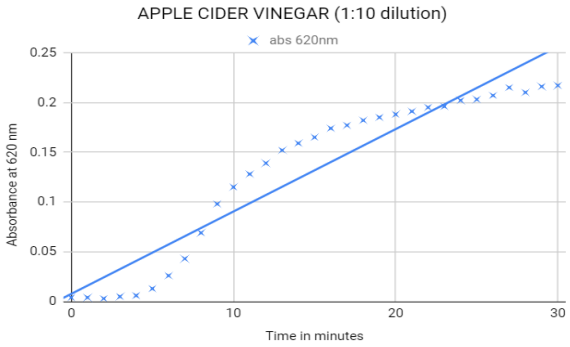
Samples	Estimation compound	Trial 1	Trial 2	Trial 3	Concentration
Wood apple	Ascorbic acid (w/w)	0.2	0.25	0.2	6.5% ± 0.5%
Apple Cider Vinegar	Acetic acid (v/v)	16.87	17	17.1	8.9% ± 1.2%

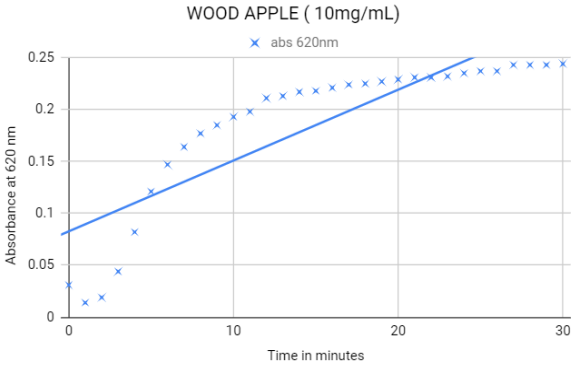
Through titrimetric analysis it was found that wood apple contained 6.5% ± 0.5% (w/w) of total Ascorbic acid in the dried pulp and ACV was found to contain 8.9% ± 1.2% (v/v) of acetic acid in it.

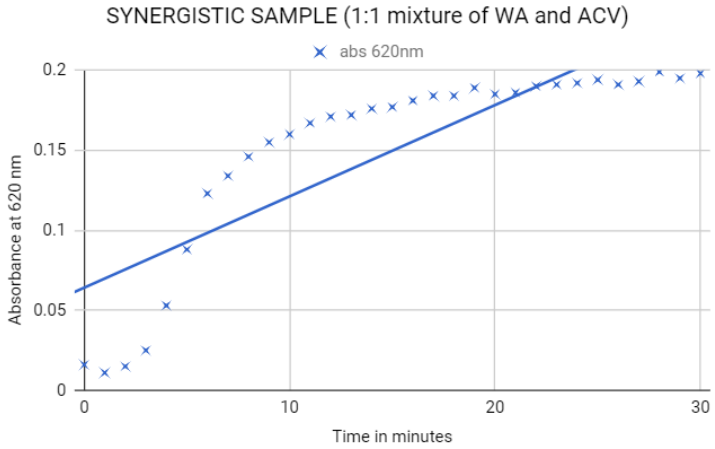
3.5. Nucleation and Aggregation Assay



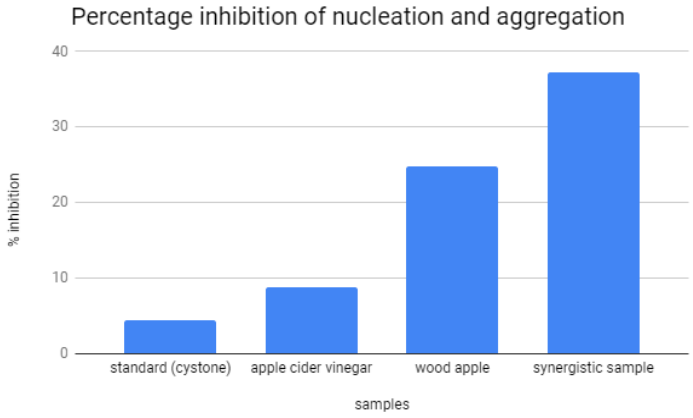








samples		slope	% inhibition
standard (cystone)	10mg/ml	0.008678629032	4.418687526
apple cider vinegar	1:10	0.008274193548	8.872902115
wood apple	10mg/ml	0.006822580645	24.86011233
synergistic sample	1:1 mixture	0.005703225806	37.18802736



From the above data, it is well understood that the nucleation and aggregation of the kidney stone is maximally inhibited by the synergistic sample (37.18802736%) i.e.,combination of wood apple and ACV followed by wood apple (24.86011233%), ACV (8.872902115%) and Standard drug (cystone - 4.418687526%).

4.DISCUSSION:

Hyperoxaluria is a major risk factor for CaOx nephrolithiasis, which in turn is associated with renal injury. High level of oxalate causes a variety of changes in the renal epithelial cells, such as an increase in free radical production and a decrease in antioxidant status, followed by cell injury and cell death [15]. These changes are significant predisposing factors for the facilitation of crystal adherence and retention [17] .Oxalate induced toxicity and free radical production are attenuated in vivo[18] and in vitro[19] by antioxidants. Phytotherapy for urolithiasis treatments is an ancient method but it has garnered the attention of researchers for its huge unexplored domain as it is an easier, cost effective alternative compared to other complicated treatment strategies.

Wood apple possess a high amount of Ascorbic acid, flavonoids, terpenoids and other minerals which contribute towards inhibiting the growth of renal calculi. Ascorbic acid content was estimated through titrimetric method based on its reducing nature and the obtained results expressed in percentage weight followed by estimation of flavonoids through colorimetric method which was expressed in Quercetin Equivalents. Similarly, total flavonoid content was estimated in ACV followed by estimation of acetic acid by titrimetric method. The obtained results were compared with the % inhibition of nucleation and aggregation shown by the respective samples. Kidney stone formation is initiated by forming small nuclear molecules of calcium oxalates which aggregate to form insoluble crystals/masses. In vitro studies of 10mg/mL methanolic extract of Wood apple showed promising results in inhibition of nucleation and aggregation of Calcium oxalate crystals. The herb extracts may contain substances that inhibit the growth of CaOx crystals. This property of plants may be important in preventing the growth of kidney stone. Aggregation may be an important factor in the genesis of stones [20,21]. 1:10 diluted ACV showed better % inhibition than the standard drug cystone and finally the synergistic sample of 1:10 diluted ACV and 10 mg/ mL methanolic extract of Wood apple (mixed in a ratio of 1:1) showed the highest % inhibition in comparison to all the other samples.

5.SCOPE OF STUDY:

This study demonstrated the possession of inhibitory properties of Wood apple and ACV towards Renal calculi nucleation and aggregation in the in vitro conditions. Also, when the two test extracts were mixed and tested for inhibitory properties, the % inhibition was shown to be the highest. This concludes that there was no antagonism in between active components of WA and ACV but in turn the samples showed better inhibition in presence of each other. Thus, proving their synergistic activity in urolithiasis.

The present work is underway and many more related activities are yet to be tested such as - Calcium oxalate crystal growth assay which checks the % inhibition of

aggregation of minerals around formed stones, Single gel diffusion Growth assay in which the ability of the sample to degrade synthetically grown stones is checked and Kidney stones degradation assay in which degradation of biologically formed (surgically removed) kidney stones in presence of test samples is tested. Further studies can be conducted to isolate the active compounds and conducting invivo studies.

6. ABBREVIATIONS:

ACV - Apple Cider Vinegar
DCPIP - 2,6 -dichlorophenol indophenol
WA - Wood apple
NaCl - Sodium Chloride
QE - Quercetin equivalents
$CaCl_2$ - Calcium Chloride
Tsc - Turbidity slope of control
$AlCl_3$ - Aluminium Chloride
Tsi - Turbidity slope of indicator
NaOH - Sodium Hydroxide
CaOx - Calcium oxalate

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