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A Comparative Study An In-Vitro Anti-Urolithiasis Activity Of Hydro-Alcoholic Seed Extract: Glycine Max L. And Achyranthes Aspera L.

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ABSTRACT

Glycine max linn, commonly referred to as soybeans, have been researched for their possible advantages in treating urolithiasis. The seeds contain chemicals, such as oxalate-degrading enzymes and phytate that can inhibit the production of kidney stones. These substances are abundant in isoflavone, flavonoid, anthocyanidin phyto-constituents, which have the ability to decrease inflammation and oxidative stress in the urinary system. Achyranthes aspera linn seeds has diuretic and anti-inflammatory characteristics, making them beneficial in traditional medicine. Research has demonstrated their ability to dissolve kidney stones, improve kidney functions, and alleviate discomfort associated with urolithiasis. The current study aimed to assess the effectiveness of both plants in laboratory methods for urolithiasis by using a crystal nucleation assay at dosages of 0.1, 1 mg, and 10 mg/ml, respectively. Glycine max linn exhibited a notable increase in the rate of crystal nucleation compared to Achyranthes aspera linn. This implies that Glycine max may have a stronger ability to promote crystal formation in a solution. The cause of this phenomenon can be described presence of bio compounds in Glycine max, which perhaps includes substances that improve nucleation processes. A Fourier Transform Infrared (FT-IR) analysis was conducted on 10-50 mg of calcium oxalate (CaOx) crystals generated during the crystal nucleation experiment. The analysis confirmed the existence within the crystal structure of calcium and oxalate ions. The results of the inquiry showed that Glycine max exhibited the highest level of crystal aggregation (56.69 %) at a concentration of 1000 mg/ml, but Sample Achyranthes aspera demonstrated the most crystal aggregation (79.26 %) at the same concentration. Calcium CaOX aggregation was most significantly inhibited by A. aspera which was very close to Cystone (80.79 %). The study examined the impact of GM extract and AA at concentrations of 0.1, 1 and 10 mg/ml on the Nucleation assay. the dimensions of the calcium oxide stone were measured using this approach and treated with AA extract at varying concentrations for varying lengths of time and GM extract also with different time intervals, when compared to a typical medication cystone. According to the Nucleation assay's results on anti-urolithiatic activity, after 270 minutes, AA extract showed a higher percentage of inhibition at a dosage of 10 mg/ml, in contrast to the prescribed medication cystone tablets However, 0.1 mg/ml of GM extract for 240 minutes, exhibited a significant % inhibition when compared to standard drug cystone. The study showed that the Achyranthes aspera linn seed extract shown a potent antiurolithiatic effect on crystal aggregation and on nucleation assay when compared to another selected seed extract of Glycine max Linn, the treatment of renal stone disease may be derived from this.

Keywords: *Glycine max linn, Achyranthes aspera linn, Crystal Aggregation and Nucleation Assay*

1. INTRODUCTION

Plants offer numerous health benefits through various means. They serve as the foundation of both medicine and phytoconstituents. Cultures around the world have used different strategies to treat illness, depending on the resources available in their specific biocultural environments. Most developing countries rely on plants for their primary healthcare. A significant number of the current pharmaceuticals employed to treat various disorders are derived from plants or plant-based therapies [1]. Traditionally, nearly all drugs were derived from biological resources. They remain vital in the present day, as 60–70% of current pharmaceuticals are derived from natural sources [2]. According to the World Health Organization

(WHO), over 80% of individuals worldwide rely on traditional medicine, with the vast majority these treatments utilizing plant extracts, as their main source of healthcare. India's traditional medical systems, including Unani, Ayurveda, Homoeopathy, and Siddha, recommend around 95% of medicines derived from plants [3].

Urolithiasis, also known as urinary calculus, is the process of developing and keeping solid non-metallic minerals (stones) in the urinary system. In the context of Ayurveda, it is referred to as Mutra-ashmari. This condition is prevalent in about with a recurrence rate of 70–80% in men and 47–60% in women, it affects 12% of the population. Calcium, especially calcium oxalate, makes up 80% of the stones at least. Urolithiasis is another name for urinary calculus, is the process of forming and collecting solid non-metallic minerals (stones) in the urinary system. In Ayurvedic nomenclature, the condition is known as Mutra-ashmari. The male recurrence rate ranges from 70% to 80%, whereas the female recurrence rate ranges from 47% to 60%. Calcium is the main component of 80% of the stones, with calcium oxalate being the primary form [4]. Although there are multiple treatments available In an effort to prevent hypercalciuria and hyperoxaluria from returning, their efficacy is diminishing [5]. The therapies consist of as an alkali-citrate and diuretic, thiazide. While extracorporeal shock wave lithotripsy and although urolithiasis treatment has been transformed by surgical endoscopic stone removal, current stone development cannot be completely prevented [6].

Glycine max linn scientifically referred to as soybeans, have been investigated for their possible advantages in the treatment of urolithiasis. Glycine max Linn seeds contain chemicals that possess potential in inhibiting the production of kidney stones. The substances mentioned consist of enzymes that degrade oxalate and phytate, which have the ability to attach to minerals present in urine and hinder their formation into stones. Furthermore, soybeans have a significant number of antioxidants and anti-inflammatory substances that have the potential to decrease inflammation and oxidative stress in the urinary tract [7].

The seeds of Achyranthes aspera linn have long been applied to traditional medicine. owing to its diuretic and anti-inflammatory characteristics. Research has demonstrated that the bioactive substances included in these seeds had the ability to dissolve kidney stones and inhibit their development. Moreover, studies have demonstrated that Achyranthes aspera seeds can improve renal function and alleviate discomfort associated with urolithiasis. Moreover, the utilization of Achyranthes aspera seeds under the guidance of a medical professional is believed to be both secure and advantageous in the treatment of urolithiasis. It is essential to bear in mind that further investigation is required to completely comprehend these seeds' modes of action and their negative effects [8, 9].

2. METHODOLOGY

The In-Vitro study of plant extract of GM and AA carried out in Aakar Biotechnologies Private Limited, Lucknow (U.P.) (Approved research lab by An ISO 9001: 2015 certified Company, Lucknow).

Plant extracts (AA and GM) was concentrated under a reduced pressure evaporator at 40C to yield a semisolid mass and that was kept in umber color glass container with tight cap and extract was kept in the refrigerator for further investigations.

Nucleation assay method [10]

Reagents

In Mumbai, India, Qualigens Fine Chemicals sold ethylene glycol. The local market was the source of Cystone (Himalaya Drug Company). Transasia Biomedicals Private Limited, Mumbai, provided the ERBA Diagnostics Mannheim GmbH, Germany, urea, creatinine, uric acid, calcium, and phosphorus estimation kits. Every remaining chemical such as Calcium chloride (50 mM), Sodium oxalate (50 mM), Tris buffer (100Mm, Ph 6.8), Sodium chloride (5M), used in the experiment were of the highest quality which is available to use.

Reagent Preparation

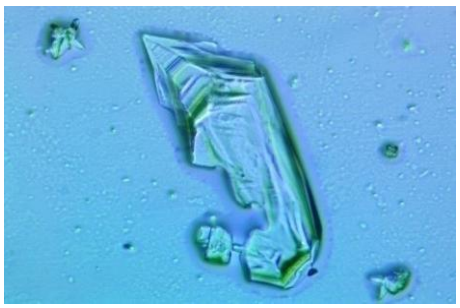
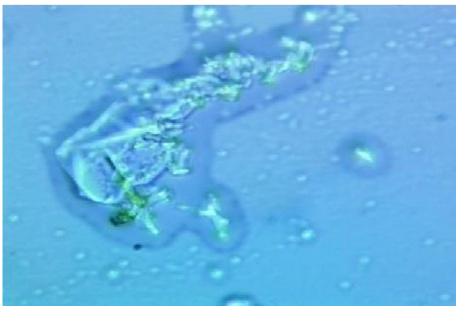
Four grams of calcium chloride were dissolved in one liter of water to create 4 mM CaCl2.Sodium oxalate (4 mM) was made by dissolving One liter of water was used to dissolve four grams of sodium oxalate. Ten grams of NaCl were dissolved in one liter of water to create 10 milliliters of NaCl.

In vitro Evaluation of Glycine max Linn seeds (GS) and Achyranthes aspera Linn seeds (AS) on Crystal Nucleation Assay [11]

With few modifications, the nucleation assay was carried out using the Rajeswari et al. (2013) methodology. At pH 6.5 and 370C, solutions of calcium chloride (90 µl) and sodium oxalate (90 µl) were made in a buffer that contained Tris and NaCl. In a 96-well plate, different concentrations of plant AA and GM extracts (0.1, 1, and 10 mg/ml) were mixed with 90 µl calcium chloride solutions. Ninety microliters of sodium oxalate were injected to start the crystallization process. Following the nucleation assay, CaOx crystals were gathered, and pictures of the crystals were taken with a Nikon Optiphot oil immersion microscope set to 1000X magnification. At 629 nm, the absorbance (optical density) was measured at various intervals of time (0, 30, 60, 90, 120, 150, 180, 210, 240, and 270 min.). A microscope was used to measure and observe the CaOx stone's size for comparison. Cystone tablets used as positive control drug solution.

Microscopic study of crystals produced during Crystal Nucleation assay

Oil immersion was used to observe With or without the extract's microscope at 1000X magnification, the CaOx crystals that developed (Nikon Opti phot) [12] as shown Figure No. 1.1 & 1.2.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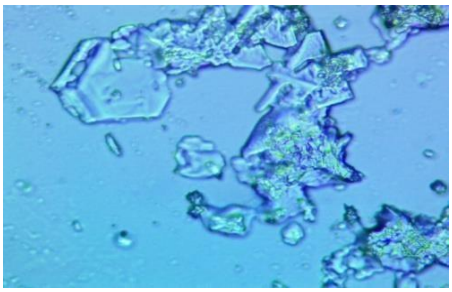	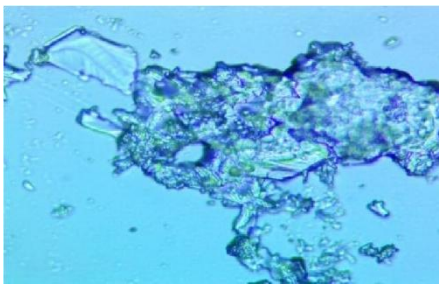
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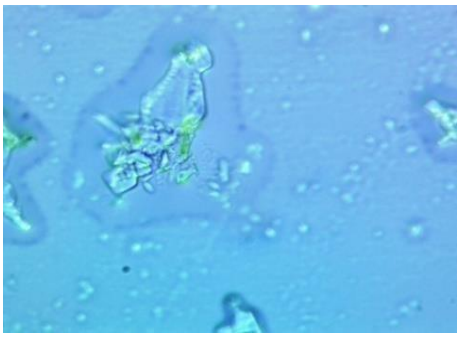
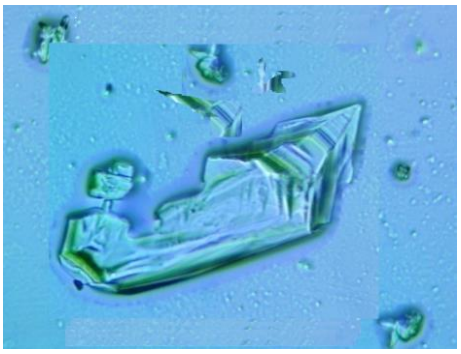
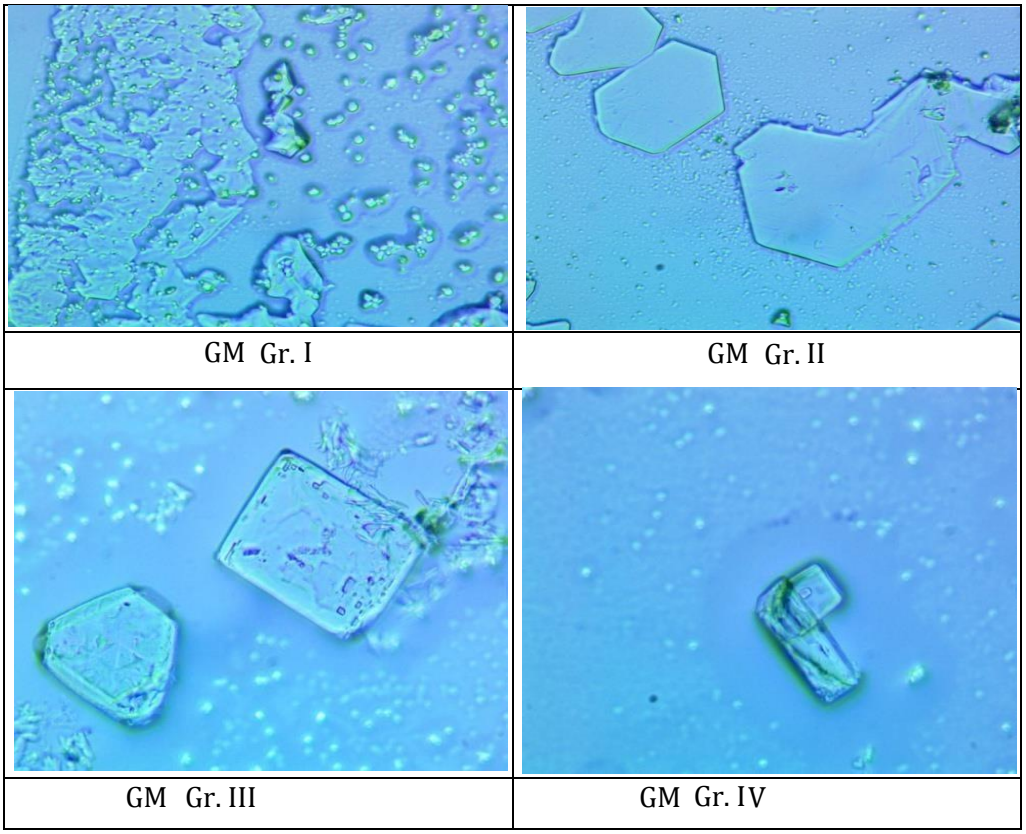
	
Gr. V	**Gr. VI**

Gr.I: Disease induced; Gr. II: 0.1mg/ml; Gr. III: 1 mg/ml; Gr. IV: 10 mg/ml; Gr. V: Cystone; Gr. VI: Control

Fig. 1.1 Urine Microscopy of Glycine max Linn extract



Gr. I: Disease induced; Gr. II: 0.1mg/ml; Gr. III: 1 mg/ml; Gr. IV: 10 mg/ml; Gr. V: Cystone; Gr. VI: Control

Fig. 1.2 Urine Microscopy of Achyranthes aspera Linn extract

[% of inhibition = [(OD CONTROL /ODTEST)/ ODCONTROL] ×100]

Where, OD CONTROL = Cystone was used as positive control

ODTEST = AA Extract and GM Extract

Nucleation is an initial step in renal stone formation. The size of the crystals reduced according to the greater hydroalcoholic extract concentrations. Thus, it was It is possible to prevent the formation and growth of CaOx crystals using the hydro-alcoholic seed of AA and GM extract.

Crystal Aggregation Assay [13]

Different concentration of Achyranthes aspera inhibited turbidity (aggregation) to a lesser extent than the control, indicating less aggregation of crystals. Concentration-dependent increases in the extracts' inhibited aggregation were observed. Aggregation is the most efficient way to increase the size, composition, and structure of urinary stones, and this process is known as calculus development.

Reagents: Calcium chloride (50Mm), Sodium oxalate (50Mm), Tris buffer (100Mm, pH 6.8), Sodium chloride (5M), Cystone (1mg/ml).

In vitro Evaluation of Glycine max Linn seeds (GM) and Achyranthes aspera seeds (AA) on Crystal Aggregation Assay

A little change was made to the methodology outlined by Atmani and Khan (2000) in order to inhibit the aggregation of the oxalate crystallization test using plant extract. To determine the inhibitory power of the AA and GM plant extracts utilized, the analysis of crystallization with and without an inhibitor is part of this framework. Through the use of the crystallization assay, different quantities of sodium oxalate (Na2C2O4) and calcium chloride (CaCl2), ranging from 50 mmol, were investigated. The final concentrations of calcium chloride and sodium oxalate were selected based on the turbidity of the solution and the sensitivity of their spectrophotometer.

In a pH 6.8 buffer comprising Tris 100 Mmol and NaCl 5 Mmol, Final concentrations of sodium oxalate and calcium chloride were prepared at 10, 50, 100, 250, 500, and 1000 µg/mL, respectively. 10 µL of herb extracts (AA and GM) at different

concentrations (10–1000 µg/mL) were mixed with a 50 mL solution of calcium chloride. 50 millimetres of sodium oxalate solution were added to initiate crystallization. A 37 °C temperature was maintained for the duration of the onehour incubation period. Using an Imark Biorad Microplate Reader, at 620 nm, the crystalline solution's optical density (OD) was determined.

The following reaction was anticipated to cause the crystals to grow:

[CaCl2 + Na2C2O4 → CaC2O4 + 2NaCl]

The percentage aggregation inhibition was then calculated using the following formula (Masao et al. 2000) by comparing the turbidity in the presence of individual AA and GM extract with that obtained in the control (Cystone). [14]

[% inhibition = (1-Turbiditysample/Turbidity control) × 100]

Results Analysis

The outcome of Glycine max Linn seeds (GM) and Achyranthes aspera seeds (AA) on Crystal Nucleation Assay

Following this formula, the relative inhibitory activity was determined: [15]

[% of inhibition = [(ODCONTROL /ODTEST)/ ODCONTROL] ×100]

Where, ODCONTROL = Cystone was used as positive control

ODTEST = AA Extract and GM Extract

Nucleation is an initial step in renal stone formation. The size of the crystals reduced according to the greater hydroalcoholic extract concentrations. Thus, it was CaOx crystal nucleation and formation could be inhibited using the hydroalcoholic seed of AA and GM extract.

Table 1.1. Nucleation assay graph of AA extract in different time-

	0 min	30min	60min	90min	120min	150min	180min	210min	240min	270min
Blank	0	0	0	0	0	0	0	0	0	0
0.1mg/ml	0.16467	0.122	0.09767	0.07367	0.211	0.341	0.358	0.29433	0.21467	0.127
1mg/ml	0.10933	0.034	0.07833	0.021	0.27767	0.30133	0.321	0.355	0.26533	0.24433
10mg/ml	0.13333	0.141	0.16067	0.07667	0.18533	0.29533	0.32267	0.29	0.201	0.308
PC	0.508	0.36167	0.261	0.18033	0.997	0.86867	0.42767	0.87833	0.627	0.97233

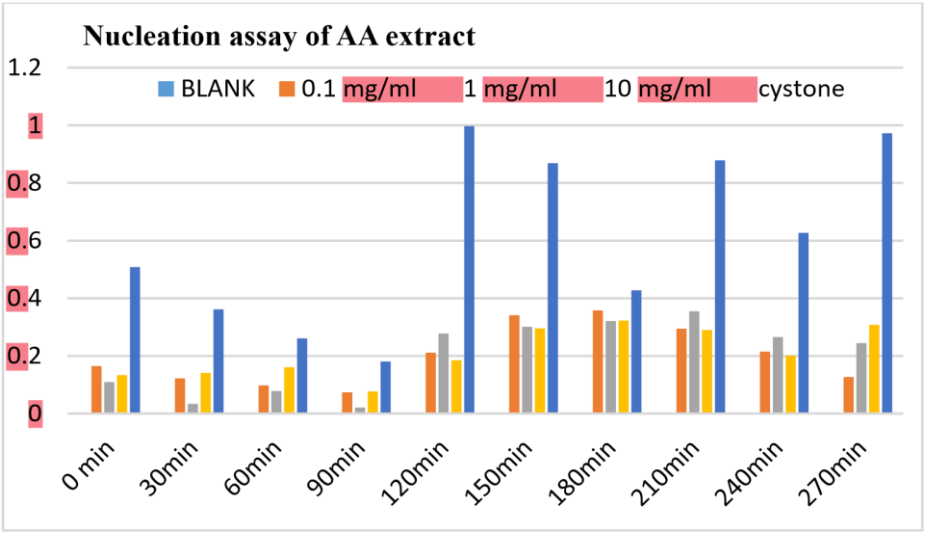


Fig. 1.3 Crystal nucleation assay of AA at different time intervals

Table 1.2 Nucleation assay graph of sample GM in different time

Time	0min	30min	60min	90 min	120min	150min	180min	210min	240min	270min
Blank	0	0	0	0	0	0	0	0	0	0
0.1mg/ml	0.092333	0.141667	0.131333	0.092333	0.151667	0.297333	0.001202	0.303	0.65132	0.000577
1mg/ml	0.040333	0.122	0.125	0.040333	0.083333	0.174	0.001202	0.307	0.269667	0.000577
10mg/ml	0.177	0.271667	0.162	0.177	0.197667	0.376	0.0938	0.303333	0.214667	0.000882
pc	0.180333	0.361667	0.261	0.180333	0.997	0.868667	0.001202	0.878333	0.626333	0.000882

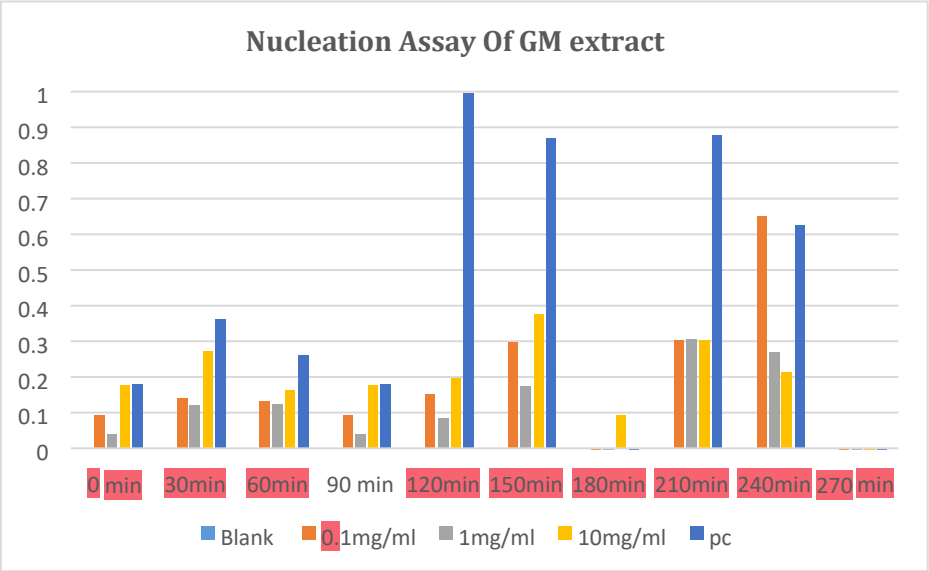


Fig. 1.4 Crystal nucleation assay of GM at different time intervals

Table 1.3 The study examined the impact of *GM* extract and *AA* at concentrations of 0.1, 1 and 10 mg/ml on the Nucleation assay. *GM* significantly increased the rate of crystal nucleation compared to *AA*.

	Blank	Cystone	Sample *GM*			Sample *AA*		
			0.1 mg/ml	1 mg/ml	10 mg/ml	0.1 mg/ml	1 mg/ml	10 mg/ml
Numbers	10-15	15-20	20-25	20-25	25-30	15-20	20-30	20-30

The results of nucleation assay under in vitro conditions of AA and GM extract using 96 well plate is presented in Fig. 1.1, 1.2 and 1.3. Under using this method, the dimensions of the calcium oxide stone were noted when treated with AA extract with varying concentrations (0.1, 1, 10 mg/ml) with different time intervals as shown in Table 1.1 and fig. 1.10 and GM extract varying in concentration (0.1, 1, 10 mg/ml) with different time intervals as shown in Table 1.2 and fig. 1.11, in contrast to the common medicine cystone. Comparing the anti-urolithiatic activity of AA extract at a dose of 10 mg/ml at 270 minutes to that of standard medicine cystone tablets, the nucleation assay showed a higher percentage inhibition. However, a dose of 0.1 mg/ml of GM extract at 240 minutes showed a considerable percentage of inhibition in comparison to the reference medicine, cystone.

Table 1.4. Effect of *Glycine max* Linn seeds (*GM*) and *Achyranthes aspera* seeds (*AA*) on Crystal Aggregation Assay

	Blank	Test	Positive control
Buffer	10		
COM crystal solution	90	90	90
Sample	0	10	0
Cystone	0	0	10

Table 1.5. Aggregation assay of sample AA extract

Test Name	Crystal aggregation Assay
Sample Extract	AA
Graph Title	Crystal aggregation Assay
X Title	Concentration (µg/ml)
Y Title	% Aggregation wrt control

Sample Conc.	**Test Replicates**			
0	0.136	0.11	0.122	0.13
10	0.113	0.119	0.115	0.117
50	0.115	0.116	0.123	0.115
100	0.107	0.103	0.101	0.103
250	0.105	0.108	0.12	0.109
500	0.106	0.113	0.108	0.111
1000	0.123	0.124	0.128	0.127
Cystone	0.092	0.1	0.106	0.107

Blank				**Corrected Values**		
0.044	0.041		0.0935	0.0675	0.0795	0.0875
0.051	0.042		0.0665	0.0725	0.0685	0.0705
0.053	0.048		0.0645	0.0655	0.0725	0.0645
0.047	0.044		0.0615	0.0575	0.0555	0.0575
0.049	0.066		0.0475	0.0505	0.0625	0.0515
0.062	0.09		0.03	0.037	0.032	0.035
0.073	0.144		0.0145	0.0155	0.0195	0.0185
0.087	0.084		0.0065	0.0145	0.0205	0.0215

Average Values		
Blank	0	
Control	0.082	

Final Values for Analysis

	Final Replicate Values			
Sample Conc.	**1**	**2**	**3**	**4**
0	-14.0244	17.6829	3.04878	-6.70732
1	18.9024	11.5854	16.4634	14.0244
10	21.3415	20.122	11.5854	21.3415
100	25	29.878	32.3171	29.878
250	42.0732	38.4146	23.7805	37.1951
500	63.4146	54.878	60.9756	57.3171
1000	82.3171	81.0976	76.2195	77.439
Cystone	92.0732	82.3171	75	73.7805

Table 1.6. Aggregation assay of sample *AA* extract at different concentration

	Stats			
Sample Conc.	Mean	SD	SEM	N
0	0 .00	13.7070856	6.8535428	4
10	15.2439024	3.14876695	1.5743835	4
50	18.597561	4.71001229	2.3550061	4
100	29.2682927	3.06903839	1.5345192	4
250	35.3658537	7.99687625	3.9984381	4
500	59.1463415	3.7916175	1.8958088	4

1000	79.2682927	2.90301969	1.4515098	4
Cystone	80.7926829	8.41227727	4.2061386	4

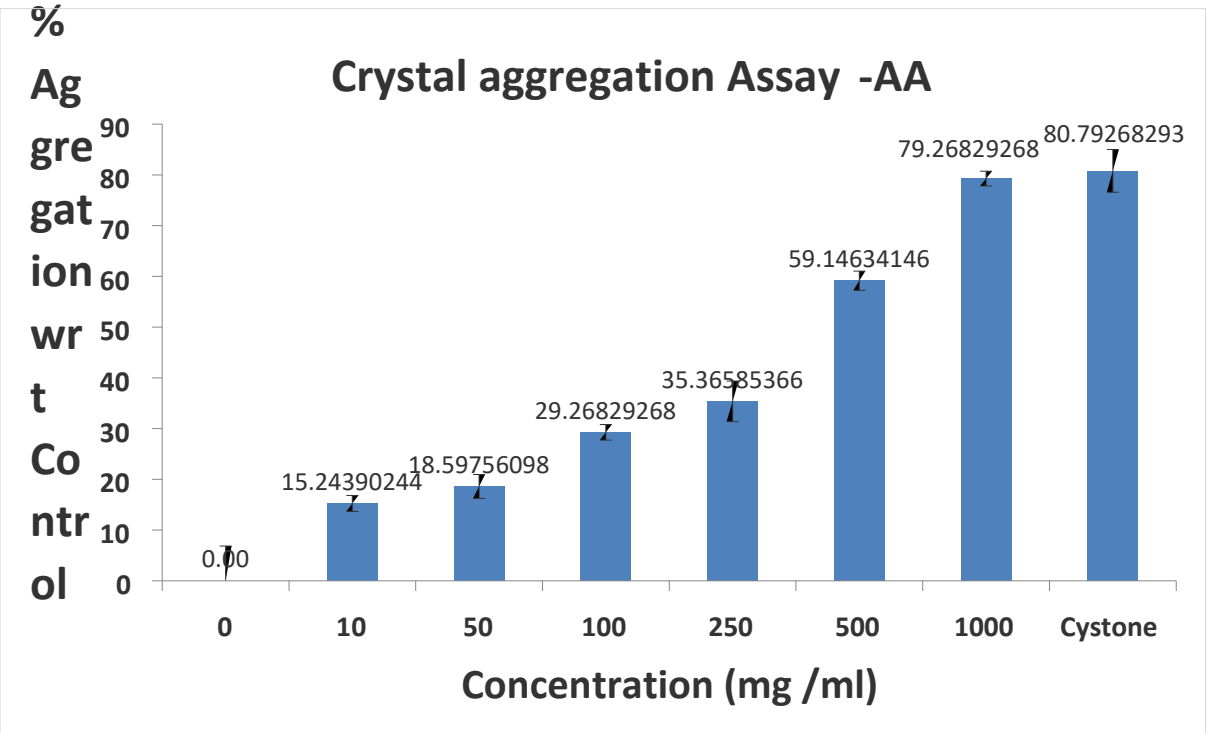


Fig. 1.5. Aggregation assay of sample *AA* extract at different concentration

Table 1.7. Aggregation assay of sample *GM* extract

Test Name	Crystal aggregation Assay
Sample Extract	GM
Graph Title	Crystal aggregation Assay
X Title	Concentration (µg/ml)
Y Title	% Aggregation wrt control

Sample Conc.	**Test Replicates**			
0	0.114	0.116	0.115	0.101
10	0.096	0.095	0.095	0.091
50	0.099	0.095	0.092	0.092
100	0.086	0.085	0.094	0.093
250	0.089	0.092	0.107	0.099
500	0.098	0.101	0.112	0.106
1000	0.107	0.104	0.118	0.127
Cystone	0.092	0.1	0.106	0.107

Blank				**Corrected Values**		
0.051	0.045		0.066	0.068	0.067	0.053
0.048	0.045		0.0495	0.0485	0.0485	0.0445
0.052	0.057		0.0445	0.0405	0.0375	0.0375
0.051	0.058		0.0315	0.0305	0.0395	0.0385
0.062	0.068		0.024	0.027	0.042	0.034
0.076	0.075		0.0225	0.0255	0.0365	0.0305
0.084	0.089		0.0205	0.0175	0.0315	0.0405
0.087	0.084		0.0065	0.0145	0.0205	0.0215

Average Values	
Blank	0
Control	0.0635

Table. 1.8. Final Values for Analysis

	Final Replicate Values			
Sample Conc.	**1**	**2**	**3**	**4**
0	-3.93701	-7.08661	-5.51181	16.5354
1	22.0472	23.622	23.622	29.9213
10	29.9213	36.2205	40.9449	40.9449
100	50.3937	51.9685	37.7953	39.3701
250	62.2047	57.4803	33.8583	46.4567
500	64.5669	59.8425	42.5197	51.9685
1000	67.7165	72.4409	50.3937	36.2205

Cystone	89.7638	77.1654	67.7165	66.1417

Table 1.9. of sample GM extract at different concentration

	Stats			
Sample Conc.	Mean	SD	SEM	N
0	0	11.0984	5.54918	4
10	24.8031	3.4919	1.74595	4
50	37.0079	5.22303	2.61152	4
100	44.8819	7.33031	3.66515	4
250	50	12.623	6.3115	4
500	54.7244	9.65437	4.82718	4
1000	56.6929	16.6165	8.30824	4
pc	75.1969	10.8631	5.43155	4

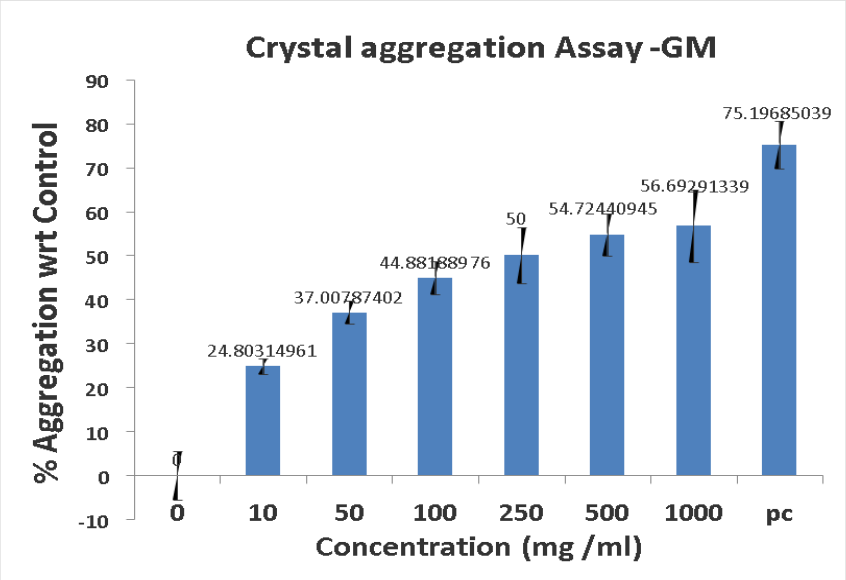


Fig. 1.6. Aggregation assay of sample *GM* extract at different concentration

3. RESULT

The study examined the impact of *GM* extract and AA at concentrations of 0.1, 1 and 10 mg/ml on the Nucleation assay. *GM* significantly increased the rate of crystal nucleation compared to *AA*. The results of nucleation assay under in vitro conditions of *AA* and *GM* extract using 96 well plate, the dimensions of the calcium oxide stone were measured using this approach and treated with *AA* extract at different dosages (0.1, 1, and 10 mg/ml) for varying lengths of time and *GM* extract of at different dosages (0.1, 1, and 10 mg/ml) with different time intervals as shown in Table 1.1, fig. 1.3. and Table 1.2 with fig. 1.4, when compared to a typical medication cystone. According to the Nucleation assay's results on anti-urolithiatic activity, after 270 minutes, *AA* extract showed a higher percentage of inhibition at a dosage of 10 mg/ml., in contrast to the prescribed medication cystone tablets However, 0.1 mg/ml of *GM* extract for 240 minutes, exhibited a significant % inhibition in contrast to the common medication cystone. Taking into account the results of the present investigation, it was discovered

that aggregation activity inhibition's effects for *AA* extract (Table 1.1 and fig. 1.3) and *GM* extracts were presented in (Table 1.2 and fig. 1.4).

Aggregation assay result showed the crystals treated with standard drug cystone, However, when the concentration of *GM* and *AA* extract was increased, the rate of inhibition increased as well. As per data analysis the *AA* extract showed a 79.26% aggregation inhibition rate at a dosage of 1000 mg/ml. in Fig. 1.5, while *GM* extract at 1000 mg/ml dose was exhibited 56.69% shown in fig.1.6. it was showed *AA* extract was more effective anti-urolithiatic activity in relation to the *GM* extract.

4. DISCUSSION

In the current investigation, the extract was greatly decreased the nucleation phase crystallization of calcium oxalate. The extracts of *GM* and *AA* prevent the crystals of calcium oxalate from aggregating. It demonstrates how phytoconstituents from plant extract influence crystal shape either directly or indirectly. The investigation's goal was to look into these crucial moments in development of CaOx stones in order to determine effectiveness of *AA* and *GM* extract as an antiurolithiatic agent.

In Nucleation assay the concentration of *AA* extract increased (10> 1> 0.1 mg/ml) with increased time intervals (0, 30, 60, 90, 120, 150, 180, 210, 240, 270 min.) it leads to more effective as compare to standard drug. As per result data fig. 1.1 at 270 min. the half-life of *AA* extract was found to be 4.5 hr., it means the *Achyranthes aspera* linn seed extract undergo patient compliance while the *GM* extract performed better result at 240 min. in 0.1mg/ml concentration. In which the half-life of *GM* extract was 4 hr., after 4 hr. the *Glycine max* linn seed extract effect will be reduced as per result obtained in fig. 1.4 that showing the patient required multiple doses at different time intervals. [13]

When a lot of crystals in a solution gather together and stick together to create massive crystal agglomerates, this process is known as crystal aggregation.[14] Aggregation is an important element in determining crystal retention because large crystal agglomerates create renal tubular obstruction, which in turn increases the development of stones. *AA* extract effectively prevented the agglomeration of CaOx crystals. are most abundance that may be present in Numerous phytochemicals are present in natural extracts of AA and GM, with a higher concentration of tannins, polyphenols, flavonoids, isoflavone, and anthocyanidins. Previous studies have also reported the diuretic, antioxidant, and CaOx crystal inhibiting qualities. [17]

In both human urine and animal models, flavonoids have been demonstrated to inhibit the crystallization of calcium oxalate. [15] as well as crystal deposition. [16]

Conclusion

Among the chosen plants, *Achyranthes aspera* Linn revealed anti-urolithiatic action in nucleation and aggregation assays that was comparable to cystone. The study's findings demonstrated that *A. aspera* seed extract was more effective in preventing crystal development and improving patient compliance. The traditional usage of Indian herbs to treat kidney disorders and urinary stones is supported by these research findings.

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