ejpmr, 2023, 10(1), 176-184

SJIF Impact Factor 6.222

EUROPEAN JOURNAL OF PHARMACEUTICAL AND MEDICAL RESEARCH

www.ejpmr.com

Research Article

ISSN 2394-3211

EJPMR

DETERMINATION OF *IN-VITRO* ANTI-UROLITHIATIC ACTIVITY OF *SPIRULINA PLATENSIS* AND *TRACHYSPERMUM AMMI*

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Article Received on 29/10/2022 **Article Revised on 19/11/2022** **Article Accepted on 09/12/2022**

ABSTRACT

Background: Kidney stones are also known as renal calculi which are formed in the kidneys due to crystal aggregation which are formed from the dietary minerals which are present in the urine. Renal calculus or crystal aggregation is a complex process which involves super-saturation, nucleation, growth, aggregation and retention of crystals within the kidney. Various in-vitro and in-vivo studies reveled that some of the phytochemical elements which are present in various plant species are useful in the management of urolithiasis or kidney stones or renal calculi. In the present study *Spirulina platensis* and *Trachyspermum ammi* were selected for screening their in-vitro anti-urolithiatic activity. **Objectives:** To isolate the active constituents from *spirulina platensis* and fruits of *trachyspermum ammi* through various extraction procedures, and to perform the anti-urolithiatic activity studies by titrimetric method of analysis, aggregation and nucleation and compare the percentage of inhibition and percent dissolution between the two herbal drug extracts. **Results and Discussion:** Both the *Spirulina* and *Ajowan (trachyspermum ammi)* extracts has shown significant level of percentage of inhibition in both the nucleation and aggregation assay, it has been observed that as the concentration (100, 200, 300, 400, 500µg/ml) of both the herbal drugs extract was increased there was significant level of decrease in the growth of the CaOx crystals *spirulina* showed more inhibition of the crystal growth than *ajowan* when compared to the standard drug cystone. In titrimetric method the percent dissolution of the *spirulina* was more than *ajowan*, and *spirulina* showed almost equal percent dissolution to standard drug cystone. **Conclusion:** From the above study *Spirulina platensis* and *Trachyspermum ammi (Ajowan)* has shown anti-urolithiatic activity by inhibiting the crystal growth, *Spirulina platensis* has shown more inhibition of crystal growth compared to *Trachyspermum ammi.*

KEYWORDS: Anti-urolithiatic Activity, Nucleation, Aggregation, Percentage Inhibition.

INTRODUCTION

Urolithiasis is the third most common disorder of the urinary tract.[1] The worldwide incidence of urolithiasis is quite high and inspite of tremendous advances in the field of medicine, there is no truly satisfactory drug for the treatment of renal calculi. Most patients still have to undergo surgery to be rid of this painful disease.[2]

Kidney stones typically form in the kidney and leave the body through the urine stream, a small stone may pass without causing any symptoms, if a stone grows more than 5millimeters (0.2 inches) it can cause blockage of the ureter, resulting in sharp and severe pain in the lower back or abdomen. A stone may also result in blood in the urine, vomiting, or painful urination.[3]

Most stones form by the combination of genetics and environmental factors. Risk factors include high urine calcium levels, obesity, certain foods, some medications, calcium supplement hyperparathyroidism, gout and not drinking enough fluids.[4]

Between 1% and 15% of people all around the globe are affected by kidney stones at some point of time in their lives. In 2015, 22.1 million cases of renal calculi occurred, resulting in about 16,100 deaths. They have become more common in the Western world since the 1970s. Generally, more men are affected with kidney stones than women.[5]

Globally, kidney stone disease prevalence and recurrence rates are increasing day by day, with limited options of effective drugs. Urolithiasis affects 12% of the world population at some stage in their life time. It affects all ages, sexes, and races but occurs more frequently in men than in women within the age of 20-49 years.[6] The relapsing rate of secondary stone formations if patients do not apply metaphylaxis is estimated to be 10-23% per

year, 50% in 5-10 year, and 75% in 20 years of the patient. In Indian population, about 12% of the population are expected to have renal stones and out of which 50% of the population may end up with loss of kidney functions.[7] Nephrolithiasis or urolithiasis is formation of urinary calculi at any level of the urinary tract. Urinary calculi are worldwide in distribution but are particularly common in some geographic locations such as in parts of the United States, South Africa, India and south-east Asia. It is estimated that approximately 2% of the population experiences renal stone disease at some time in their life with male- female ratio of 2:1. The ultimate incidence is observed in 2nd to 3rd decades of life. Renal calculi are characterized clinically by colicky pain (renal colic) as they passdown along the ureter.[8]

There are four main types of urinary calculi they are.
1.Uric acid stones: These are the most common types of stones, seen in children, these are frequently opaque calcium stones.[9] These are formed because of the deposition of urine in the urinary tract either in the pelvic, urinary bladder or the ureter. Approximately about 6% of the urinary stones are made of uric acid.

2. Calcium Oxalate stones: Calcium Oxalate stones are the most common type of stones that can be diagnosed and comprises about 75% of all the kidney stones. They may be pure stones of the calcium oxalate (50%) or calcium phosphate (5%) or the mixture of calcium oxalate and calcium phosphate (45%).[10]

3.Struvite stones: About 15% of the urinary stones are made of magnesium-ammonium-calcium phosphate, often they are called struvite, hence mixed stones are also called as struvite stones or triple phosphate stones. These stones are formed as a result of infection of the urinary tract with urea-splitting organisms that produces ureases such as by species of proteus, klebsiella, pseudomonas and enterobacteria.[11]

4.Cystine stones: The diseased state of 'cystinuria' is the main cause for the formation of cystine stones around the urinary tract. Larger content of cystine oozes into the urine and the stone form because of the leek.[12]

However, these stones can pass out through the urinary system by intake of plenty of water, surgery is not really necessary, these stones can be prevented by change in the lifestyle such as drinking more amount of fluids, avoiding dairy products which contains calcium constituents, calcium pills as well as foods with added vitamin D and certain antacids that has calcium base and less intake of meat also helps in avoiding the formation of kidney stones.[13]

The stones can be treated by various other procedures such as medical therapy, surgery, Extracorporeal shockwave lithotripsy (ESWL), Percutaneous Nephrolithotomy, Ureteroscopic stone removal etc.[14]

Spirulina platensis is a non-toxic, blue green algae which is filamentous and used as a cyanobacteria taken by the human as dietary food.[15] It is primarily obtained from two species of cyanobacteria: *Arthrospira platensis*, *Arthrospira Maxima* and *Arthrospira fusiformis* belonging to the family microcoleaceae. It is a biomass which is the dried form of *arthrospira platensis*. This blue- green algae is the primary diet in humans, animals and aquatic life. It has been proven that it boosts the immune system, and reduce the risk of many cancers and infections.[16] The biological properties of *Spirulina platensis* includes anti-cancer, Antimicrobial, Antioxidant, Antiviral, Antidiabetic, and immune-stimulant effects.[17] C-phycocyanin, a molecule which contains phycocyanobilin, a homolog of biliverdin, is one of the major protein constituent which is present in *Spirulina.*[18]

Trachyspermumammi L. belonging to family Apiaceae a highly valued medicinal plant. The root is diuretic in nature and the seeds possess excellent aphrodisiac properties. The seeds contain 2- 4.4% of brown coloured oil known as Ajwain oil.[19] The main component of this oil is thymol, which is used in treating gastrointestinal ailments, lack of appetite and bronchial problems. Ajowan is traditional potential herbs, is widely used for its therapeutic effect for various diseases in humans and animals such as flatulence, atonic dyspepsia and diarrhea. The seed of ajowan acts as anthelmintic, carminative, laxative, and stomachic, cures abdominal tumors, abdominal pains and piles and bears anti-inflammatory and anti-oxidant activity.[20]

In present study we have studied weather *spirulina platensis* and *trachyspermum ammi* possess anti-urolithiatic by compare it to the standard drug cystone which is mainly used in the treatment of kidney stones.

MATERIALS AND METHODS

The Spirulina platensis powder was collected from the ayurvedic store at Kukatpally. The fruits of *Trachyspermum ammi linn.* (Ajwain) were collected from local store at KPHB Hyderabad.

The plants were identified and authenticated from Osmania University, Department of Botany. The Voucher has been preserved at Pharmacognosy Department, Malla Reddy Pharmacy College, Hyderabad. The collected powder and fruits were made free from extraneous matter air- dried. The dried fruits were powdered and stored in well closed container.

EXTRACTION

The dried powdered of spirulina platensis and fruits of ajowan were subjected to extraction. The powdered samples were extracted with methanol and water in the ratio of 70:30 maceration process for 7 days. The container was agitated from time to time. After 7 days, the container was warmed on water bath at a temperature

of 30 ± 5ºC for 10 min to ensure better extraction. The contents were filtered and concentrated under reduced pressure using rotary flash evaporater. The resultant concentrated extracts were further dried in dessicator and stored in air tight container. The extracts were weighed and percentages of the yields were calculated in terms of air-dried weight of the powder of *spirulina platensis* and fruits of ajowan.

QUALITATIVE PHYTOCHEMICAL SCREENING

The qualitative phytochemical investigation of ajowan and spirulina showed the presence of alkaloids, glycosides, phenols, flavonoids, tannins and steroids.

Phytochemical test	Ajowan fruits extract	Spirulina platensis
Alkaloids:		
Dragendroff test	+	+
Mayer'test	+	+
Hager's test	+	+
Wagner's test	+	+
Phenols & Tannins:		
Ferric chloride test	+	-
Gelatin test	+	-
Steroids/Triterpenoids:		
Salkowski's test	+	+
Liebermann-Burchard test	+	+
Saponins		
Foam test	-	-
Flavonoids:		
Lead acetate test	+	+
Shinoda test	+	+
Alkaline reagent test	+	+
Proteins:		
Millon's test	+	+
Biuret test	+	+
Amino acids:		
Ninhydrin test	+	+
Carbohydrates:		
Molish's test	-	-
Fehlings test	-	-
Benedict's test	-	-
Barfoed's test	-	-
Glycosides:		
Borntragers test	+	+
Legals test	-	+
Killer killani test	+	+

EVALUATION OF ANTI-UROLITHIATIC ACTIVITY BY TITRIMETRIC METHOD[21,22]

The basis for the evaluation for in-vitro anti-urolithic of spirulina and fruit of ajowain is to avoid the pain and sacrifice of experimental animals. The activity was performed in three steps.

Step-1: preparation of calcium oxalate stones.

Equimolar solution of calcium chloride dihydrate in 100 ml distilled water and sodium oxalate in 100 ml of 2N sulfuric acid was dissolved (Fig.1). Calcium chloride solution was added to the sodium oxalate solution drop wise with proper stirring. The resulting precipitate was calcium oxalate. The precipitate was freed from traces of sulfuric acid by ammonia solution (Fig.2). Washed with distilled water and dried at 50- 60ºC for 4 hours. The calcium oxalate precipitate prepared by this method resembles the kidney stones (Fig.3).

Fig.1

Fig.2

Fig.3

Step-2: Preparation of Semi-permeable membrane.

The semi-permeable membrane is prepared from eggs. The membrane is lies in between the outer calcified shell and the inner contents like albumin and yolk. Shell was removed chemically by placing the eggs in 2 M HCl for an overnight (Fig.4), which caused complete decalcification (Fig.5). Further, washed with distilled water, and carefully with a sharp surgical blade a small cut was made on the membrane and the contents squeezed out completely. Then the membrane washed thoroughly with distilled water, and placed it in ammonia solution for less than 10 minutes in the moistened condition and rinsed it with distilled water. The membrane were stored in the refegirator (10-15ºC), in phosphate buffer (pH 7-7.2) till the day of estimation of calcium oxalate (Fig.6).

Fig.4(egg in 2M HCL)

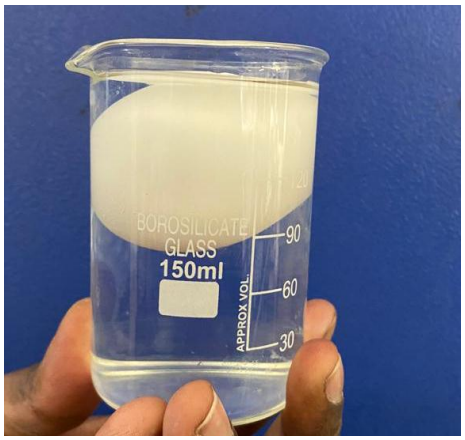


Fig.5(decalcified egg)

Fig.6(semi-permeable memb)

Step-3: Estimation of Calcium oxalate amount of Titrimetry.

To estimate the amount of calcium oxalate by titrimetry total 4 groups were used. Weighed exactly 1 mg of the calcium oxalate and 10 mg of the extracts/standard and packed it together in the permeable membrane in evaluation (Fig.7, Fig.8) This was allowed to suspend in a conical flask containing 100 ml phosphate buffer (Fig.9).

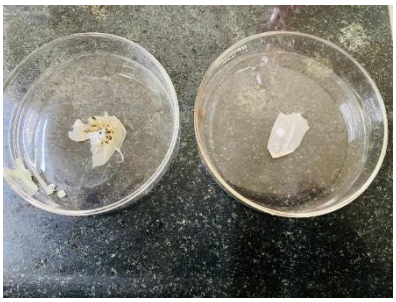


Fig.7


Fig.8


Fig.9

Group-I: served as negative control; contains only 1 mg of calcium oxalate.
Group-II: tested for the anti-urolithic activity for standard herbal drug cystone
; 1 mg of calcium oxalate + 10 mg of cystone
Group-III: tested for the ani-urolithic activity of spirulina extract; 1 mg of calcium oxalate + 10 mg of spirulina extract.
Group-IV: tested for the anti-urolithiatic activity of ajowan extract; 1 mg of calcium oxalate + 10 mg of ajowan extract.


Fig.10


Fig.11

The conical flask of all groups was preheated to 37ºC for 2 hours and incubated at the room temperature for about 24 hours (Fig.10). The contents of semi-permeable membrane from each group were collected into a test tube and 2 ml of 1 N sulfuric acid added. The contents of the dish were titrated with 0.9494 N KMnO4 till a light color observed as an end point (Fig.11).
1 ml of 0.9494 N KMnO4 equivalent to 0.1898 mg of calcium oxalate.

The amount of undissolved calcium oxalate is subtracted from the total quantity used in the experiment in the beginning, to know how much quantity of calcium oxalate actually test substance could dissolve.

Amount of undissolved calcium oxalate = W1-W2
Where,
W1 = weight of initial calcium oxalate;
W2 = weight of utilized calcium oxalate.

EVALUATION OF ANTI-UROLITHAITIC ACTIVITY BY AGGREGATION AND NUCLEATION ASSAY[23,24,25]
Instruments: Digital balance, UV spectrophotometer.
Apparatus: Spatula, beaker, conical flask, tripod stand, chinadish, aluminium foil.

Procedure
Aggregation Assay: Cacl2 and Na2C2O4 solutions (50 mmol each) were mixed together, heated to 60˚C in a water bath for 1 hour, then incubated overnight at 37˚C to prepare seed of CaOx crystals. After drying CaOx crystal solution (0.8mg/mL) was prepared in a 0.05 mol/l Tris-HCL and 0.15mol/l Nacl buffer (Ph6.5) one milliliter of aliquots (100-500µg/ml) of the sample was added to 3ml CaOx solution, vortexed and then incubated at 37˚C for 30 min. Optical density (OD) of the final mixtures was the read at 620 nm wavelength and percent incubation og aggregation was the calculated for the standard polyherbal drug (Cystone).
% inhibition = {1- (Test OD/Control OD)} X 100

Nucleation Assay: Calcium chloride (CaCl2) (5 mmol/l) and sodium oxalate (Na2C2O4) solution (7.5 mmol/l) were prepared in Tris-HCL (0.05 mol/l). Dilutions of 100-500µg/ml were prepared in with distilled water and one milliliter of each sample concentration was mixed with 3 ml CaCl2 solution followed by the addition of 3ml Na2C2O4 solution. Final mixtures were incubated for 30 mins at 37˚C. The Optical density (OD) of the mixture was the measured at by comparing to the calculated standard polyherbal drug (Cystone)
% inhibition = {1- (Test OD/Control OD)} X 100

RESULTS AND DISCUSSIONS:

- **Titrimetric method of analysis of cystone, *spirulina platensis* and *trachyspermum ammi.***

Table-1.

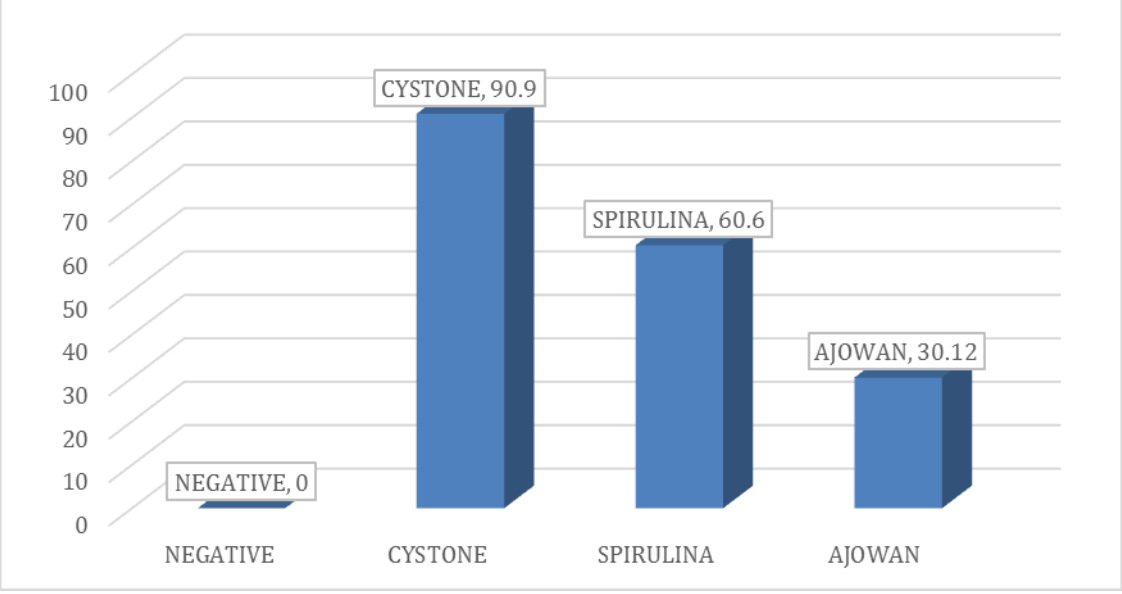
GROUP	BURETTE READING (ml)	AVERAGE	WT.OF CALCIUM ESTIMATED	WT.OF CALCIUM REDUCED	%DISSOULTION
GROUP-1 (NEGATIVE)	0.6 0.7 0.7	0.68	0.1328	0	
GROUP-2 (CYSTONE)	0.09 0.1 0.2	0.12	0.01896	0.12	90.9
GROUP-3 (SPIRULINA)	0.3 0.3 0.3	0.3	0.05694	0.08	60.6
GROUP-4 (AJOWAN)	0.45 0.5 0.48	0.49	0.0949	0.04	30.12

- **Percentage dissolution of cystone, *spirulina platensis* and *trachyspermum ammi***

Table-2

GROUP	%DISSOLUTION
GROUP-1 (NEGATIVE)	0
GROUP-2 (CYSTONE)	90.9
GROUP-3 (SPIRULINA)	60.6
GROUP-4 (AJOWAN)	30.12

- **Graph representing percentage dissolution of cystone, *spirulina platensis* and *trachyspermum ammi(ajowan)***



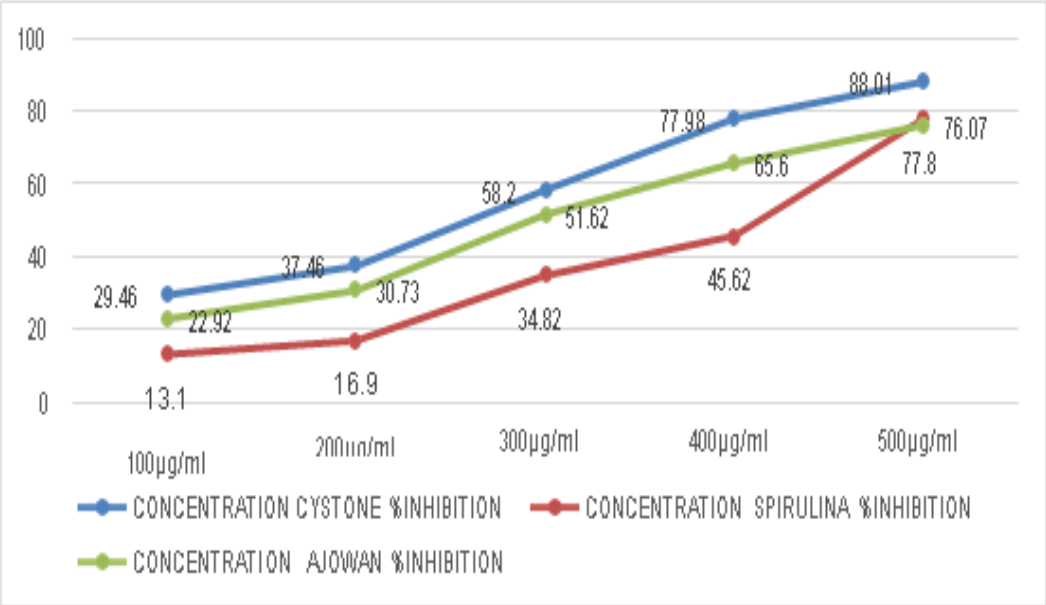
Graph-1

- **Table showing effect of *spirulina platensis* and *trachyspermum ammi(ajowan)* on calcium oxalate crystals by aggregation assay.**

Table-3.

Concentration	Cystone		*Spirulina platensis*		*Trachyspermum ammi*	
	OD	% Inhibition	OD	% Inhibition	OD	% Inhibition
100µg/ml	0.326	29.46	0.402	13.1	0.3562	22.92
200µg/ml	0.289	37.46	0.384	16.9	0.3201	30.73
300µg/ml	0.1923	58.2	0.3012	34.82	0.2236	51.62
400µg/ml	0.1018	77.98	0.2513	45.62	0.1590	65.6
500µg/ml	0.0554	88.09	0.1026	77.8	0.1106	76.07

- **Graph representing effect of *spirulina platensis* and *trachyspermum ammi* on calcium oxalate crystals by aggregation assay**



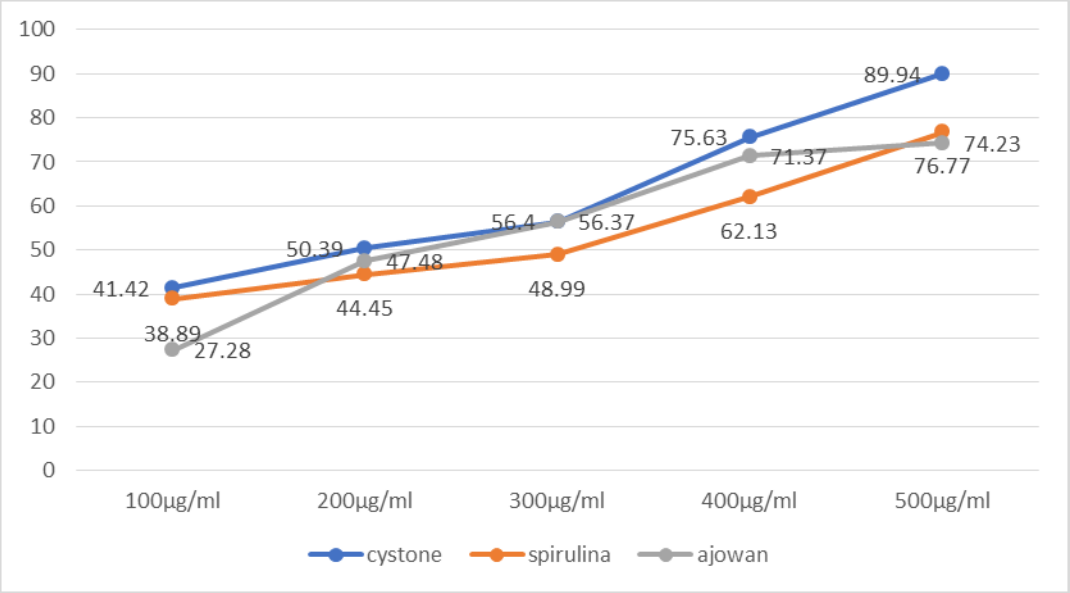
Graph-2

- **Table representing effect of *spirulina platensis* and *trachyspermum ammi* on calcium oxalate crystals by nucleation assay.**

Table-4.

Concentration	Cystone		*Spirulina platensis*		*Trachyspermum ammi (ajowan)*	
	OD	% Inhibition	OD	% Inhibition	OD	% Inhibition
100µg/ml	0.116	41.42	0.121	38.89	0.144	27.28
200µg/ml	0.0982	50.39	0.110	44.45	0.104	47.48
300µg/ml	0.0863	56.4	0.101	48.99	0.0864	56.37
400µg/ml	0.0482	75.63	0.075	62.13	0.0567	71.37
500µg/ml	0.0199	89.94	0.046	76.77	0.0506	74.23

- **Graph representing effect of *Spirulina platensis* and *Trachyspermum ammi* on calcium oxalate crystals by nucleation assay**



Graph-3.

CONCLUSION

The present work demonstrates that the extracts of ajowan fruits and spirulina are good source of various phytochemicals like alkaloids, glycosides, phenols, flavonoids, tannins and steroids. This study evaluates that anti-urolithic activity of the extracts of *ajowan* fruits and *spirulina platensis.*

The work was performed by using in vitro anti-urolithiatic activity model for calculating percent dissolution of calcium oxalate which rescembles the kidney stones. From the results of the study spirulina platensis and *trachyspermum ammi* fruits possess anti-urolithiatic property. The percent dissolution of calcium oxalate with spirulina extract was almost equal to the standard drug cystone

In the Aggregation Assay and Nucleation Assay the percent inhibition of turbidity increased with the increase in the concentration of both the extracts but spirulina has showed highest percentage of inhibition in both the Aggregation Assay (77.8%) and Nucleation Assay (76.77%) at 500µg/ml concentration than standard cystone and ajowan extract.

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