



Evaluation of Antiulithiatic Activity of *Asparagus Racemosus* on *In Vitro* Calcium Oxalate Crystallization Methods

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Abstract: The primary purpose of this research was to interrogate the antiulithiatic impact of the aqueous root extract of *Asparagus racemosus* (AEAR) on *in vitro* crystallization methodology. AEAR is generally known as Shatavari, belongs to Asparagaceae family and it consists of various phytochemical components, such as alkaloids, amino acids, proteins, steroids, saponins, flavonoids, tannins, phenols and carbohydrates. The antiulithiatic activity was studied in the availability and lack of AEAR at the concentration range of 100-1000 µg/ml by accessing crystal nucleation, crystal aggregation, and crystal growth assay methods. Standard drug Cystone was made use of positive control in the concentration range of 100-1000 µg/ml. The percentage inhibition frequency of crystal nucleation, crystal aggregation and crystal growth by AEAR and standard drug cystone was recognized to be dose-dependent in nature. The percentage inhibition of crystal nucleation, crystal aggregation, and crystal growth of cystone and AEAR was found to be 82.31±7.26 % and 65.62±5.66 %, 67.90±3.47 % and 59.54±2.13 %, 72.04±4.46 % and 59.15±4.12 %, respectively. The half maximal inhibitory concentration (IC₅₀) estimates of the standard drug cystone on crystal nucleation, crystal aggregation and crystal growth were computed to be 415.30±21.35, 573.7±65.53 and 566.20±62.06 µg/ml, respectively, while the AEAR, IC₅₀ values were reported to be 689.60±17.16, 818.10±60.56 and 760.30±71.07 µg/ml, respectively. The current results of the *in vitro* crystallization analysis demonstrated that an aqueous root extract of *Asparagus racemosus* presents calcium oxalate crystal inhibition potential on crystal nucleation, crystal aggregation, and crystal growth specified as a significant and promising antiulithiatic function.

Keywords: *Asparagus racemosus*, Crystal nucleation, Crystal aggregation, Crystal growth, Cystone, and Antiulithiatic activity

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Received On 17 July 2020

Revised On 27 August 2020

Accepted On 09 September 2020

Published On 04 December 2020

Funding This research did not receive any specific grant from any funding agencies in the public, commercial or not for profit sectors.

Citation Kishore Bandarapalle, Prasanna Raju Yalavarthi and Chandra Sekhar Kothapalli Bannoth , Evaluation of Antiulithiatic Activity of *Asparagus Racemosus* on *In Vitro* Calcium Oxalate Crystallization Methods.(2020).Int. J. Life Sci. Pharma Res.10(5), P56-62
<http://dx.doi.org/10.22376/ijpbs/lpr.2020.10.5.P56-62>

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I. INTRODUCTION

Calculi deposition in the kidney is one of the prehistoric and most prevalent disorders in history of mankind.¹ Urolithiasis originates from the Greek words “ouron” (urine) and “lithos” (stone). It is known to be the third most severe urinary tract condition.² Calcium oxalate calculi are prevalent and periodically recorded calculi among distinct types of calculi. Calcium oxalate calculi are managed to accessible in two forms stated calcium oxalate monohydrate (COM) and calcium oxalate dihydrate (COD). The COM type of calcium oxalate has a higher affinity in contrast with COD to the binding of renal tubular epithelial cells. The COD type is usually seen in the urine of healthy subjects.³⁻⁴ Renal pathogenesis entail sequence of actions including, supersaturation, crystal nucleation, aggregation, retention and growth.⁵ Urine supersaturation is an initial step that contributes to the eventual development of solid crystal particles and facilitates crystallization in the urine. This also helps in crystal nucleation, and then these crystals consolidate into crystal aggregates. The resultant aggregates devastate the renal tissue and are deposited and accumulated to the progress of the calculi formation.⁶⁻⁷ Further, the findings demonstrated that oxalate influenced renal damage originates by the association of reactive oxygen species (ROS) in urolithiasis.⁸⁻¹⁰ Urolithiasis may therefore be deterred by obstruction of essential steps in the crystallization process accompanied by ROS-influenced renal disruption. Medical, surgical treatments or combinations of medical and surgical strategies occasionally practice in the administration of urolithiasis. The choice of treatment is dependent solely on size, position, and type of calculi. Ureteroscopy and extracorporeal shock wave lithotripsy and percutaneous nephrolithotomy are established operations for the removal of calculi, particularly if the size of the calculi exceeds 1 cm. Such methods are relatively expensive, invasive, increase the risk of retreatment and trigger undesirable side effects, like hemorrhage, hypertension, tubular necrosis, and resulting renal fibrosis contributing to cell injury, and the recurrence of the calculus is very common. The treatment of synthetic drugs relies on the type of calculi and is correlated involving adverse effects and recurrence.¹¹ Therefore, a large gap was constructed to fill the treatment approach that would fulfill the criteria of economic, clinically divergent risk factors, minimized risks, and recurrence levels. Interest is also concentrated on overseeing the ailment in the early stages of the construction of the calculi instead of the dumping efforts in the treatment preceding the emergence of the calculi which have extensively identified the recurrence impact. Numerous medicinal plants have been known for the treatment of urinary calculi since ancient times. In the current situation, the global population depends on medicinal plants for their various pharmacological activities, reducing risks, side effects, cost-effective and readily accessible. The main purpose of the current research is to investigate the antiurolithiatic behavior of the plant by addressing the inhibition of the initial stages in the development of calculi utilizing a well-known *In vitro* crystallization model. This approach examines the efficacy of plant extract at the early stages of the development of the calculi using crystal nucleation, crystal aggregation and crystal growth assay. *Asparagus racemosus* is generally known as shatavari and belongs to the Asparagaceae family. In English: Wild asparagus, Telugu: Pilli gaddalu; Gujarati: Satavari; Hindi: Satavari, Satavari, Satmuli; Kannada: Shatavari; Malayalam: Satavari; Marathi: Asvel, Shatmuli, Satavari; Oriya: Chhotaru,

Mohajolo, Sotabori; Punjabi: Bozidan, Satawar; Tamil: Tannirvittan, Nirmittan, Ammaikodi; Sanskrit: Satamuli, Satapadi, Shatavari. The Root of *A. Racemosus* draws attention because it consists of various phytochemical components, such as alkaloids, amino acids, proteins, steroids, saponins, flavonoids, tannins, phenols and carbohydrates.¹² It is extensively used for anti-inflammatory, analgesic, antipyretic, diuretic, hepatoprotective, antimicrobial, antioxidant, strong wound healer, anti-rheumatic, anticancer, antidiabetic and anti-infective properties.¹³ Thus, in the current research, the antiurolithiatic behavior of the aqueous root extract *A.racemosus* (AEAR) at distinct phases of calculi development was explored through the application of an *in vitro* crystallization model.

2. MATERIALS AND METHODS

2.1 Chemicals

Analytical standard chemicals (Sigma Aldrich, Merck India Ltd., and Hi-media) purchased from Bros Scientifics, Tirupati, India, were used in the present interrogation. Cystone, (Himalaya Drug Company, Bangalore, India) was purchased from Apollo Pharmacy, Tirupati.

2.2 Plant Material

Roots of *A. racemosus* were collected from the Sri Srinivasa Ayurvedic Pharmacy, Tirupati. It was identified and certified by Botanist Dr. K. Madhava chetty, Assistant professor, Department of botany, Sri Venkateswara University, Tirupati. Voucher specimens (voucher No: 0698) were submitted to the Sri Padmavathi School of Pharmacy, Tiruchanoor. The dried root was coarsely crushed and prepared for extraction.

2.3 Preparation of aqueous Extract of *A. racemosus*

The 100 g of coarsely crushed root powder macerated with 500 ml of distilled water at room temperature for 48 h.¹⁴ The extract was concentrated, and the earned semisolid mass of 13 g was preserved in an airtight container free from moisture, heat, and air and coded as AEAR.

2.4 Preliminary Phytochemical Screening

AEAR was pre-screened for availability of alkaloids, glycosides, terpenes, anthraquinones, tannins, saponins, phenols, flavonoids, sterols and carbohydrates utilizing standard protocols.

2.5 *In vitro* CaOx crystallization model

2.5.1 Crystal Nucleation assay

The solutions of 7.5 mM of sodium oxalate and 5 mM of Calcium chloride solutions were prepared using buffer consisting of 0.05 M/L of tris aminomethane hydrochloride (Tris-HCl) and 0.15 M of sodium chloride at pH 6.5. Calcium chloride solution of 8 ml was blended simultaneously with 1 ml AEAR at varied concentrations of 100, 200, 400, 600, 800 and 1000 µg/ml. Crystallization was stimulated by the insertion of 1 ml of sodium oxalate solution and the absorbance shift was recorded at 620 nm in a UV spectrophotometer (UV- 1800, Shimadzu Pvt. Ltd.) for 30 minutes at 37°C. The procedure was followed for the control, substituting distilled water instead of the extract. All

samples were inspected in triplicate. Standard drug Cystone was used as a positive control for comparison at distinct concentrations including 100, 200, 400, 600, 800 and 1000 $\mu\text{g}/\text{ml}$. The percentage inhibition of nucleation rate was then

$$[(I - (T_{\text{Si}} / T_{\text{Sc}})) \times 100]$$

However T_{Si} was the nucleation turbidity slope in the existence of an inhibitor sample, i.e., cystone/AEAR and T_{Sc} was the nucleation turbidity slope in the absence of the inhibitor (control).

2.5.2 Crystal Aggregation assay

The severity of the crystal aggregation of CaOx was determined by the procedure of Atmani and Khan with meager adjustments.¹⁶⁻¹⁷ The COM crystals were produced by combining 50 mM solutions of sodium oxalate and calcium chloride. The solutions were tailored to 60 °C in a water bath, cooled to 37 °C and held overnight. The solution was then centrifuged and evaporated at 37°C. CaOx crystals

accomplished by comparing the turbidity slope of the varying concentrations of cystone or AEAR with the control by the succeeding formula.¹⁵

were used at a desired concentration of 0.8 mg/ml, articulated with a buffer containing 0.05 M of Tris-HCl and 0.15 M of sodium chloride at pH 6.5. The test was tracked at 37°C in the existence and absence of AEAR at distinct concentrations of 100, 200, 400, 600, 800 and 1000 $\mu\text{g}/\text{ml}$. The absorbance was determined for one hour for every 10 minutes time duration at 620 nm. All experiments were performed in triplicates. Cystone was used as a positive control. Percentage inhibition of aggregation intensity was then computed by contrasting the turbidity slope of varied concentrations of cystone/AEAR with the turbidity slope of the control i.e., without inhibitor (cystone/AEAR) by the ensuing formula.

$$[(I - (T_{\text{Si}} / T_{\text{Sc}})) \times 100]$$

Where T_{Si} was the turbidity slope of aggregation in the existence of inhibitor samples, i.e., cystone/ AEAR and T_{Sc} was the turbidity slope of aggregation in the absence of inhibitor.

2.5.3 Crystal Growth assay

The crystal growth assay is presented on the basis of the frame work documented by Nakagawa et al. with few requisite modifications.¹⁸⁻¹⁹ COM stone slurry 0.2 mg/ml was processed with 50 mM sodium acetate buffer of pH 5.7. Calcium chloride 1 mM and sodium oxalate 1 mM were formulated with a buffer comprising 10M of Tris-HCl and 90 mM of NaCl was regulated to pH 7.2. COM crystal seed (0.2 μl) was subjected to the solution typically consisting of 1 mM of calcium chloride and 1 mM of sodium oxalate. The abundance of free oxalate reduced with the advent of COM slurry owing to the initiation of the consumption of oxalate. The deterioration in free oxalate was gauged by

spectrophotometry at a wavelength of 214 nm. In order to examine the inhibitory effect of AEAR on CaOx crystal growth one ml at distinct concentrations of 100, 200, 400, 600, 800 and 1000 $\mu\text{g}/\text{ml}$ was applied to the above described COM slurry containing calcium chloride and sodium oxalate and cystine was used as a positive control. The similar procedure was retreated for the control by supplanting distilled water in place of the AEAR/cystone. All experiments were inspected in triplicate. The relative reduction rate of free oxalate was ascertained using the baseline value and the value after 30 seconds in gestation with or without cystone or AEAR. The relative percentage inhibition of crystal growth was computed as follows.

$$[(C - I)/C] \times 100$$

Where I is the relative rate of degradation of free oxalate in the presence of the inhibitor sample, i.e., cystone/ (AEAR), C is the relative rate of degradation of free oxalate without any inhibitor sample.

3. STATISTICAL ANALYSIS

All outputs were expressed as Mean Standard deviation (SD) of triplicate findings. The 50% inhibitory concentration (IC_{50}) value was calculated by logistic regression analysis by utilizing Graph pad prism software version 5.0.

4. RESULTS AND DISCUSSION

Phytochemical analysis discovered the existence of alkaloids, glycosides, terpenes, anthraquinones, tannins, saponins, phenolic compounds, flavonoids, sterols, and carbohydrates in the aqueous extract of *Asparagus racemosus*.

4.1 Effect of AEAR on crystal nucleation

Percentage inhibition of crystal nucleation of standard drug

cystone and AEAR at distinct concentrations 100, 200, 400, 600, 800 and 1000 $\mu\text{g}/\text{ml}$ boosted from $25.28 \pm 3.16\%$ to $82.31 \pm 7.26\%$ and $18.43 \pm 4.82\%$ to $65.62 \pm 5.66\%$ respectively (Table 1). Cystone and AEAR were reported to have dose-dependent inhibition of crystal nucleation. The IC_{50} values of cystone and AEAR on crystal nucleation were reckoned to be 415.30 ± 21.35 and $689.60 \pm 17.16\text{ }\mu\text{g}/\text{ml}$, respectively (Graph 1).

4.2 Effect of AEAR on crystal aggregation

Similar dose dependent implications were witnessed in the crystal aggregation assay. Percentage inhibition of crystal aggregation of cystone and AEAR was accessed as $28.77 \pm 3.18\%$ to $67.90 \pm 3.47\%$ and $10.54 \pm 2.26\%$ to $59.54 \pm 2.13\%$ respectively (Table 1) and the IC_{50} values of

cystone and AEAR were ascertained to be 573.70 ± 65.53 and 818.10 ± 60.56 $\mu\text{g/ml}$, respectively (Graph 2).

4.3 Effect of AEAR on crystal growth

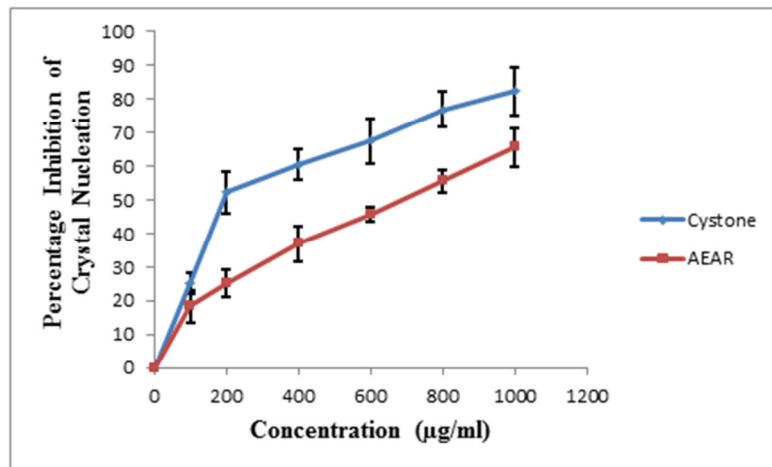
A significant escalation in the percentage inhibition of crystal growth was noticed in existence of cystone and AEAR at diverse concentrations in ascending sequence elevated from

28.08 ± 2.13 % to 72.04 ± 4.46 % and 16.82 ± 3.52 % to 59.15 ± 4.12 % respectively and it was signified decrease of free oxalate levels in the presence of cystone and AEAR (Table I). The IC_{50} values of cystone and AEAR on crystal growth were found to be 566.20 ± 62.06 and 760.30 ± 71.07 $\mu\text{g/ml}$ respectively (Graph 3).

Table I Consequence of AEAR on In vitro calcium oxalate crystallization

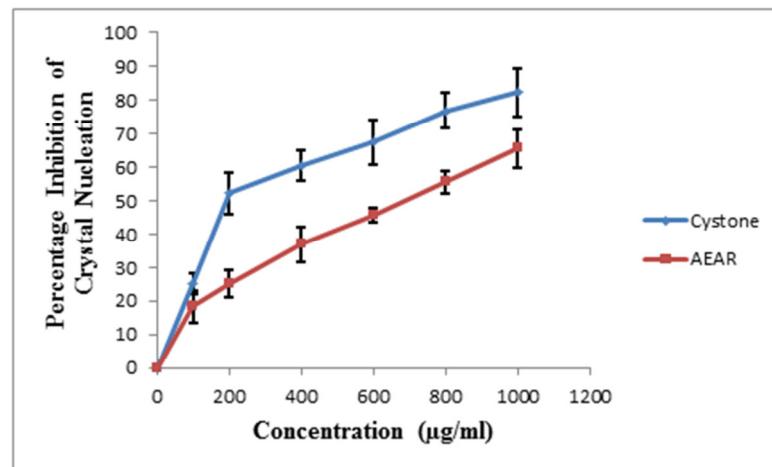
Concentration ($\mu\text{g/ml}$)	Percentage Inhibition of crystal nucleation		Percentage Inhibition of crystal aggregation		Percentage Inhibition of crystal growth	
	Cystone	AEAR	Cystone	AEAR	Cystone	AEAR
100	25.28 ± 3.16	18.43 ± 4.82	28.77 ± 3.18	10.54 ± 2.26	28.08 ± 2.13	16.82 ± 3.52
200	52.30 ± 6.23	25.03 ± 4.13	35.18 ± 6.12	22.64 ± 3.42	37.31 ± 3.37	27.54 ± 2.37
400	60.52 ± 4.68	37.98 ± 3.66	47.29 ± 3.44	32.05 ± 1.24	45.45 ± 6.72	34.73 ± 5.89
600	67.49 ± 6.84	45.57 ± 2.23	56.26 ± 2.76	38.31 ± 4.34	54.54 ± 2.93	42.60 ± 3.96
800	76.96 ± 5.17	55.54 ± 3.34	63.10 ± 4.86	47.86 ± 3.67	63.22 ± 4.18	52.91 ± 2.72
1000	82.31 ± 7.26	65.62 ± 5.66	67.90 ± 3.47	59.54 ± 2.13	72.04 ± 4.46	59.15 ± 4.12
IC 50	415.30 ± 21.35	689.60 ± 17.16	573.70 ± 65.53	818.10 ± 60.56	566.20 ± 62.06	760.30 ± 71.07

All the outputs are represented as mean \pm SD of 3 observations. IC_{50} was calculated from the logistic regression analysis, AEAR: Aqueous root extract of *A.racemosus*, and SD: Standard deviation.



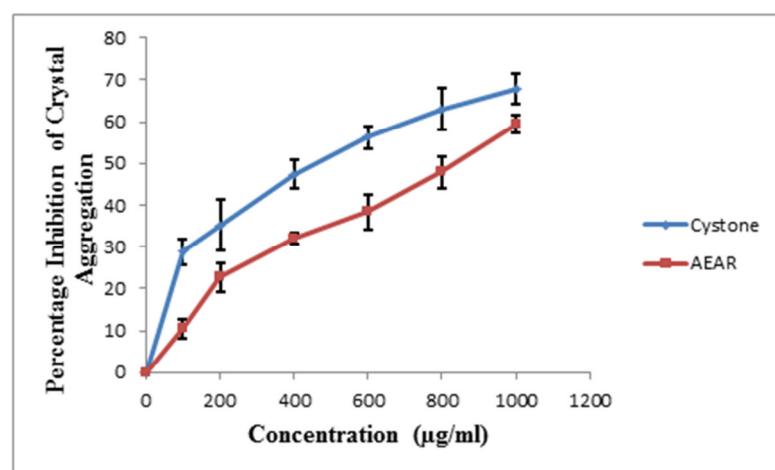
The values are represented as mean \pm S.D of 3 observations. SD: Standard deviation.

Graph 1. Consequence of AEAR on crystal nucleation



The values are represented as mean \pm S.D of 3 observations. SD: Standard deviation.

Graph 2. Consequence of AEAR on crystal aggregation



The values are represented as mean \pm S.D of 3 observations. SD: Standard deviation.

Graph 3. Consequence of AEAR on crystal growth

5. DISCUSSION

Urolithiasis has left a challenging task in its management with multifactorial etiology and increased incidence of recurrence. From the past few decades, the research involves monitoring the basic steps involved in the development of calculi.²⁰ Crystal nucleation is the main phase in a supersaturated solution leading to the transition of the liquid to the solid state. The first ever formed crystal nucleus has grounded surfaces to build a crystal lattice; the resulting crystal diameter is enhanced by the inclusion of additional components or clusters in the nucleus. These crystals assemble in order to build broad structures, called crystal aggregates, which are the key stage in the evolution of calculi.²¹ In the current *in vitro* study, the percentage inhibition of CaOx crystallization on nucleation, aggregation and growth was examined in the presence of AEAR and standard cystone. Results illustrate the successful inhibition of crystal nucleation, crystal aggregation, and crystal growth which plays a crucial role in calculi emergence. It was reported that the inhibition of the distinct methods is dose-dependent, i.e. the percentage inhibition increased with an increase in concentration in the range of 100-1000 μ g/ml in both AEAR and standard drug cystone.²²⁻²³ Though AEAR inhibitory activity was relatively low compared to the reference drug cystone, it was found to be successful in inhibiting CaOx crystallization. Inhibition of crystallization by AEAR involved distinct mechanistic points of view were implicated according to investigations of earlier reports. The major reason involved in crystal inhibition process is preventing the development of COM form of crystal by entailing the modifications resulting in formation of faulty or unstable COD crystal form which have less affinity to renal tubular cells and easily gets excreted.²⁴⁻²⁵ The another mechanism include forming soluble complex of the phytochemical constituents of AEAR with the insoluble calcium salts.²⁶ These findings provide a provisional affirmation of the antiurolithiatic performance of *Asparagus racemosus*, and assists in the processing of further research for effective establishment as an antilithiatic agent.

6. CONCLUSION

The current investigation has ensured the antiurolithiatic activity of aqueous root extract of *Asparagus racemosus* in *vitro* calcium oxalate crystallization techniques with the statistical evidence of inhibition of calcium oxalate crystallization. Further *in vivo* studies are recommended to investigate the mechanism of action and the phytoconstituents accountable for antiurolithiatic activity.

7. ACKNOWLEDGEMENTS

The first author wishes to express deepest gratitude to the Management and Dr. D. Ranganayakulu, M. Pharm., Ph.D., Principal, Sri Padmavathi School of Pharmacy, Tiruchanoor, Andhra Pradesh, India, for presenting all the necessary laboratory demands of the research and constant support. The first author is thankful to Dr. C. Sridhar, M. Pharm., Ph.D., Professor, Dept. of Pharmaceutical analysis, Sri Padmavathi school of pharmacy, Tiruchanoor, Andhra Pradesh, India, Dr. D. Sujatha, M. Pharm., Ph.D., Assistant Professor, Institute of pharmaceutical technology, Sri Padmavati Mahila Visvavidyalayam, Tirupati, Andhra Pradesh, India, Dr. V. Shanmugam, M. Pharm., Ph.D., Dept. of Pharmaceutics, Sri Padmavathi school of pharmacy, Tiruchanoor, Andhra Pradesh, India, and Dr. N. Sree Lakshmi, M. Pharm., Ph.D., Dept. of Pharmacology, Gokaraju Rangaraju college of pharmacy, Hyderabad, Telangana, India, for their valuable inputs and support.

8. AUTHORS CONTRIBUTION STATEMENT

Kishore Bandarapalle, structured and carried out the experiments, developed the designs and interpreted the data. Prasanna Raju Yalavarthi, Chandra Sekhar Kothapalli Bannoth, engaged in the organizing and execution of the project on the processing of the experimental data, drafted the manuscript and surveyed the findings of the work.

9. CONFLICTS OF INTEREST

Conflict of interest declared none.

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