ORIGINAL ARTICLE

In vitro antiurolithiatic activity of c-phycocyanin isolated from *Spirulina platensis*

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Abstract

Background: Pashanabheda is used as antiurolithiatic in Ayurveda. In the present study, *Spirulina platensis* was selected for isolation of its active constituent, C-Phycocyanin (C-PC), and screening for *in vitro* antiurolithiatic potentials. **Objective:** Screening of compound isolated from *S. platensis* for antiurolithiatic potentials. **Materials and Methods:** The algae sample was subjected to preliminary analysis. Then, the sample was processed for the extraction of phycocyanin from *S. platensis* by various extraction methods, purified and then characterization was performed by reverse phase high-pressure liquid chromatography and by mass spectrometry. Finally, *in vitro* antiurolithiatic activity was screened by nucleation and aggregation assay. **Results:** The isolated C-PC exhibited inhibitory action in both nucleation and aggregation assays to significant level. In the aggregation assay gradually decrease in the calcium oxalate (CaOx) crystal nucleation as well as growth was observed by light microscopy. The findings of the nucleation assay indicate that phytoconstituents inhibited the crystallization of CaOx in solution. There were less and smaller particles with increasing concentration of the phycocyanin. The increasing concentrations of C-PC (100, 200, 300, 400, and 500 µg/ml) inhibited the CaOx crystal growth. C-PC demonstrated slightly better results compared to cystone standard solution to inhibit the formation of CaOx dihydrate crystals in the nucleation assay. **Conclusion:** The isolated C-PC has shown antiurolithiatic effect by significantly reducing the size and growth of calculi in the kidneys in the *in vitro* assays.

Key words: aggregation assay, C-Phycocyanin, *in vitro* antiurolithiatic activity, nucleation assay

INTRODUCTION

Urinary calculi are the third most prevalent disorder of the urinary system. Approximately 80% of these calculi are composed of calcium oxalate (CaOx).[1-4] Urine is normally a supersaturated solution and only some individuals are prone to this disease. One reason for this is the presence of inhibitors of lithogenesis in urine, including macromolecules, citrate, and magnesium.[4,5] Thus, an imbalance between the promoters such as low urine volume, calcium, oxalate, uric acid, phosphate, and inhibitors may represent a potential factor in lithogenesis.

Nowadays, stone formation is the oldest and serious painful urologic disease with significant prevalence in the population due to change in lifestyle and dietary factors. Stone formation or lithiasis is characterized by calculi formation. It has two main types such as nephrolithiasis and urolithiasis. Calculi formation in urinary bladder, ureter, or any part of urinary tract rather than kidney is known as urolithiasis while nephrolithiasis is characterized calculi formation in kidney.[6-10] In general, calcification for the formation of bone and teeth takes place in controlled biological situations. Uncontrolled pathological crystallization occurs when solvent becomes supersaturated leading to the formation of precipitates in the body called as kidney stones.

Extracorporeal shock wave lithotripsy (ESWL) and drug treatment revolutionized urological practice almost became the standard procedure for eliminating kidney stones. However, shock waves had traumatic effects, residual stone fragments persisted and infection could occur. Moreover,

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Received: 09-12-2020
Revised: 11-02-2021
Accepted: 24-02-2021

ESWL may cause acute renal injury, a decrease in renal function, hemorrhage, and hypertension. Therefore, it is worthwhile to look for alternative means such as medicinal plants or phytotherapy.[11,12] Data from *in vitro*, *in vivo* studies and clinical trials reveal that phytotherapeutic agents could be useful as either an alternative or an adjunctive therapy in the management of urolithiasis.[13-15]

Oxalic acid is biosynthesized from ascorbic acid, glycolate, and glyoxylate in the metabolism of higher plants. A significant loss of minerals is more prevalent in the body when it is consumed in large content of oxalate rich foods. When calcium ions present in the body bind with free oxalic acid/oxalate, it precipitates as insoluble crystals.[12]

A large number of plants have been used in India since ancient times, which claim the efficient cure of urinary stone. C-Phycocyanin (C-PC), a phycobiliprotein, is a water-soluble and colored protein component of the photosynthetic light-harvesting antenna complexes present in cyanobacteria, red algae, and cryptomonads. Due to its anti-oxidant, anti-inflammatory, hepatoprotective, and radical-scavenging, lipid lowering, anti-arthritic properties C-PC are widely used in food, cosmetics, and pharmaceuticals. Spirulina, a type of cyanobacteria, has a high growth rate with as much as 25% (w/w) C-PC content in the biomass, and thus is considered as a promising and commercial C-PC source.[14-16] Therefore, present study intends to establish the scientific rationality of the antiurolithiatic activity of C-PC isolated from *Spirulina platensis* by *in vitro* methods.

MATERIALS AND METHODS

Collection and authentication of plant

S. platensis is blue – green algae was purchased from Parrys Nutraceuticals, Chennai, Tamil Nadu, India, and was authenticated by Dr. V. Sampath Kumar (Scientist D in-charge), Botanical Survey of India, Southern regional center, Coimbatore, Tamil Nadu, India.

Extraction methods[14-16]

Distilled water extraction

10 g of *Spirulina* biomass was suspended in the distilled water and kept in the deep freezer at −20°C for 24 h and then centrifuged at 6000 rpm for 10 min.

Homogenization

10 g of the wet biomass was homogenized with the help of mortar and pestle in the presence of glass beads in 50 mM of sodium phosphate buffer (pH 6.8).

The extract was kept at −20°C for 24 h and centrifuged at 6000 rpm for 10 min. The pellet was re-extracted with buffer to ensure complete recovery of phycocyanin.

Freezing and thawing

Phycocyanin was extracted by repeated freezing and thawing of cells by which the wet biomass (10 g) was mixed with 50 mM sodium phosphate buffer (pH 6.8) and kept at −20°C for 24 h. The above procedure was repeated 4 times. Then, the sample was centrifuged at 6000 rpm for 10 min. Then, the supernatant was taken for phycocyanin estimation.

Acid extraction

10 g of biomass was taken in two test tubes, in each test tube, 2 and 5 ml of the concentrated hydrochloric acid was added. The samples were kept in room temperature for 24 h. Samples were centrifuged at 6000 rpm for 10 min, and then supernatant was taken for phycocyanin estimation.

Heating

10 g of the wet biomass was taken in a test tube and kept inside the water bath, which was set at a temperature of about 60°C for 10 min. The sample was centrifuged at 1000 rpm for 10 min and supernatant was taken for phycocyanin estimation.

Every 1 h freezing and thawing

10 g of the wet biomass was taken in the beaker along with the 50 mM sodium phosphate buffer and kept at −20°C for 1 h and thawed at 4°C for 1 h. In the same way, 3–6 cycles were repeated .Then, the biomass was centrifuged at 6000 rpm for 10 min and supernatant was taken for phycocyanin estimation. Amount of C-PC was measured as described by Bennett and Bogard (1973) and purity was determined by using the formulae: Purity = A620/A280.

Purification of C-PC

The C-PC was extracted from blue-green algae by various methods.

Among the six methods, C-PC extracted by repeated freezing at −20°C and thawing at room temperature was found to be effective method. Cell debris was removed by centrifugation at 5000 rpm for 10 min and the extract thus obtained was termed as crude extract. Amount of C-PC was measured and purity was determined using the formulae:

Purity = A620 / A280.
Extraction yield = (C-PC) V/DB

Where yield is the extraction yield of C-PC in mg C-PC/dry biomass (g), V is the solvent volume (ml), and DB is the dry biomass (g). The optical density (OD) values were tabulated.[17,18]

Characterization of the C-PC

Reverse phase high-pressure liquid chromatography (HPLC)

To characterize the C-PC, reverse phase HPLC was performed using C5 column. PDA detector set at 620 and 226 nm. The absorption spectra of these two chromatogram peaks were critically analyzed.[19]

Mass spectrometry (liquid chromatography [LC] MS /MS)

The MS has been regarded as one of the most important analytical tool in studies of drug metabolism and biochemical toxicology. With the commercial introduction of new ionization methods such as API techniques in combination of LC-MS/MS, it has now become a truly indispensable technique in research. Triple stage quadrupole and ion trap MS are presently used for this purpose, because of their sensitivity and selectivity. It showed maximum identity with C-PC of *S. platensis*.[20]

In vitro antiurolithiatic activity

Aggregation assay

The rate of aggregation of the CaOx crystals was determined by a spectrophotometric assay with slight modifications. The CaOx monohydrate (COM) crystals were prepared by mixing both the solutions of calcium chloride and sodium oxalate of 50 mM each. Both solutions were then equilibrated. The solutions were then cooled to 37°C and then evaporated. The COM crystals were then dissolved with 0.5 ml of 0.05 mM Tris buffer and 0.5 ml of 0.15 mM NaCl solution at pH 6.5 to a final concentration of 1 mg/ml. Absorbance at 620 nm was recorded. The rate of aggregation was estimated by comparing the slope of turbidity in the presence of the extract against control.[20,21]

Nucleation assay (turbidity method)

The inhibitory activity of the extracts on the nucleation of CaOx crystals was determined by a spectrophotometric assay. Crystallization was initiated by adding 100 µl of 4 mM calcium chloride and 100 µl of 50 mM sodium oxalate solutions to 0.5 ml of human normal urine, both prepared in a buffer containing 0.5 ml of 0.05 mM Tris buffer and 0.5 ml of 0.15 mM NaCl solution at pH 6.5 and 37°C and adjusted to volume by adding 1.5 ml of distilled water. The rate of nucleation was determined by comparing the induction time of crystals (time of appearance of crystals that reached a critical size and thus became optically detectable) in the presence of the extract and that of the control with no extract. The OD was recorded at 620 nm, and the percentage inhibition calculated as (1-OD (experimental)/OD (control))/100.[22-24]

RESULTS AND DISCUSSION

Extraction of C-PC

The C-PC was extracted from blue-green algae by various methods [Table 1]. Among the six methods, C-PC extracted by repeated freezing at −20°C and thawing at room temperature was found to be effective method. Cell debris was removed by centrifugation at 5000 rpm for 10 min and the extract thus obtained was termed as crude extract. Amount of C-PC was measured and purity was determined using the formulae:

Purity = A620/A280.

C-PC = (A615)–(0.475 × A652)/5.34.

Extraction yield = (C-PC) V/DB.

Where yield is the extraction yield of C-PC in mg C-PC/dry biomass (g), Vis the solvent volume (ml), and DB is the dry biomass (g). The OD values were tabulated.

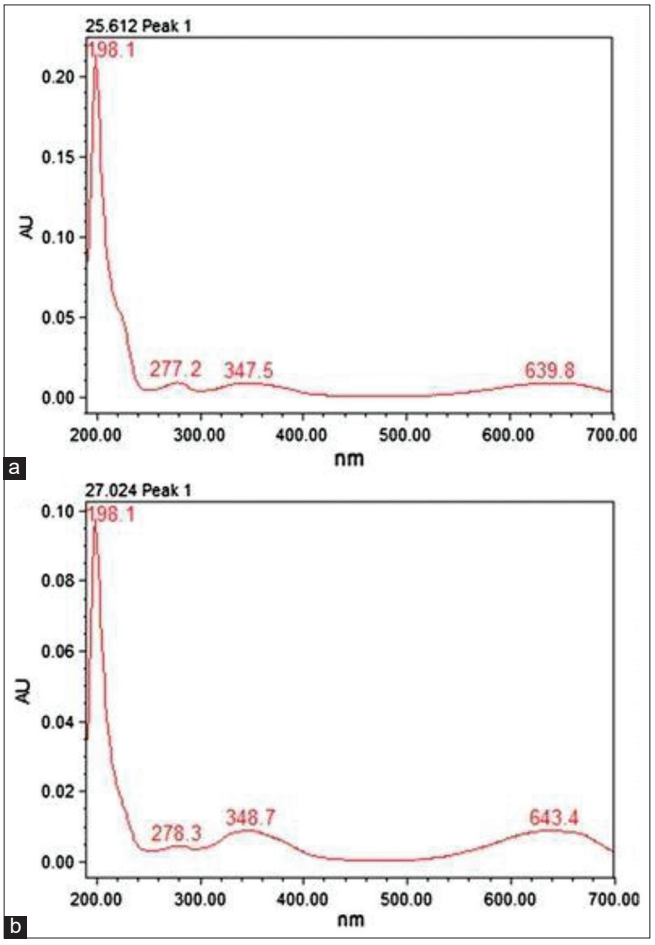

Figure 1: Reverse phase high-pressure liquid chromatography profile of C-Phycocyanin from *Spirulina* strain using PDA detector (620 nm) showing α and β subunit peaks

Table 1: Extraction of C-Phycocyanin

Method	pH	Temperature	Optical density (OD)	
			A620	A280
Concentrated HCl extract	1	Room temperature	-	-
Normal freezing and thawing extract	6	–20°C	2.682	3.521
Homogenized freezing and thawing extract	7	–20°C	2.765	3.468
Water extract	6	–20°C	2.872	3.612
1 h freezing and thawing extract	6	–20°C	2.582	3.716

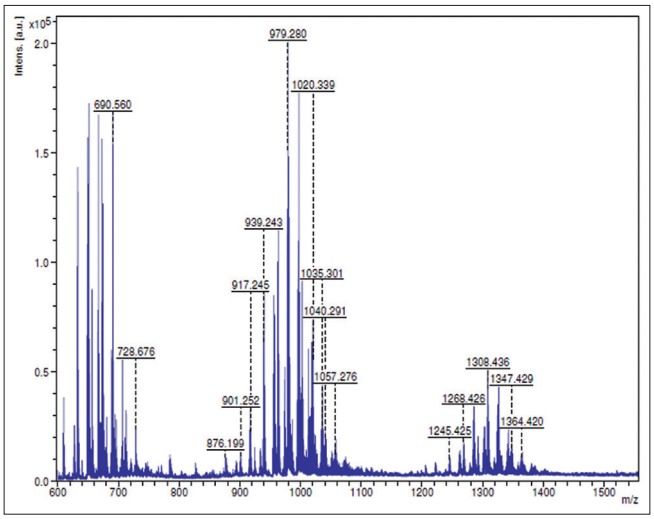

Figure 2: Mass spectrophotometer results

Purification of C-PC

To characterize the C-PC, reverse phase HPLC was performed using C5 column [Figure 1]. PDA detector set at 620 and 226 nm revealed two major peaks at 25.612 and 27.024 min. When absorption spectra of these two chromatogram peaks were critically analyzed, it was found that A620:A280 for the first peak RT = 25.612 min; which was approximately 1. It is due to the presence of one phycocyanobilin (PCB) chromophore, thus indicating that this peak corresponds to α subunit of PC, while the A620:A280 for the second peak RT = 27.024 min; it was approximately 2, which is due to the presence of two PCB chromophores, and therefore this peak corresponds to *β* subunit of PC.

Characterization of the C-PC by MS (LC MS/MS)

The MS has been regarded as one of the most important analytical tool in studies of drug metabolism and biochemical toxicology. With the commercial introduction of new ionization methods such as API techniques in combination of LC-MS/MS, it has now become a truly indispensable technique in research. Triple stage quadrupole and ion trap MS are presently used for this purpose, because of their sensitivity and selectivity. It showed maximum identity with C-PC of *S. platensis* [Figure 2].

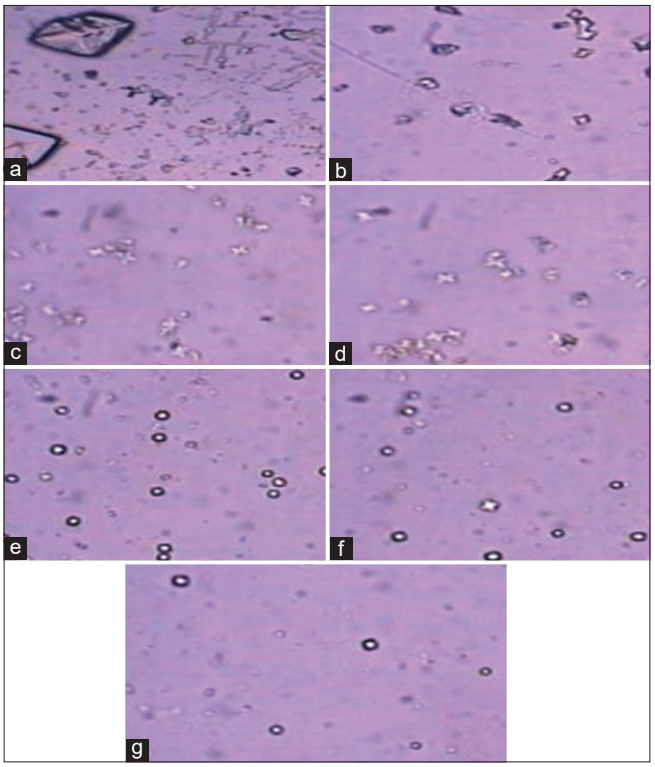

Figure 3: Calcium oxalate (CaOx) crystals, observed under light microscope (×100), formed in the metastable solution of CaOx in the absence (a) which are larger and the presence of saponin rich fraction of C-Phycocyanin (b) 10 mg/ml, (c) 20 mg/ml, (d) 40 mg/ml, (e) 60 mg/ml, (f) 80 mg/ml, and (g) 100 mg/ml gradually decreases the CaOx crystals nucleation as well as growth

Aggregation assay

CaOx crystals begin grow; aggregate with other crystals and retained in the kidney. This is aggregation process which causes renal injury. C-PC demonstrated slightly better compared to cystone standard solution to inhibit promoted the formation of COD crystals[25] [Figure 3].

Nucleation assay

C-PC inhibited the crystallization by inhibiting nucleation of CaOx through disintegrating into smaller particles with increasing concentrations of the fraction. From the results

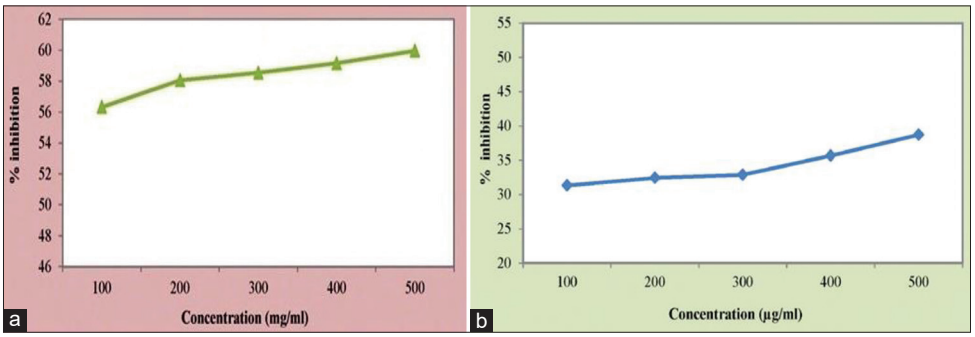

Figure 4: Effect of cystone and C-Phycocyanin on calcium oxalate (CaOx) crystal nucleation (a) effect of cystone on CaOx crystal nucleation (b) effect of C-Phycocyanin on CaOx crystal nucleation

of the nucleation assay confirmed that the extract contained nucleation preventing agents [Figure 4].

DISCUSSION

Kidney stones are reportedly affecting mankind since long time and have been one of the causes for renal failure.[24,26] As there is no single effective drug available for urolithiasis today, surgery is considered to be the best option especially when other alternatives fail. However, it is expensive and not affordable for common man. Hence, the natural drugs are considered to be next alternative. Pashanabheda plants are a group of medicinal plants which are used in Indian traditional medicinal system by Ayurveda practitioners as antiurolithiatic drugs and *S. platensis* is used conventionally as both antiurolithiatic and diuretic.

In the aggregation assay, CaOx crystals begin to grow; aggregate with other crystals, and retained in the kidney.[27] This is aggregation process which causes renal injury. C-PC demonstrated slightly better compared to cystone standard solution to inhibit promoted the formation of COD crystals. COM has a stronger affinity with cell membranes; it may lead to become higher potential risk for renal calculi formation.

An *in vitro* crystallization study was performed, since nucleation is an important first step for the initiation of crystals, which then grow and form aggregates. The main findings of the present study were that cyanin rich fraction from plants inhibited the crystallization by inhibiting nucleation of CaOx in solution; less and smaller particles were formed with increasing concentrations of the fraction. The results of the nucleation assay confirmed that the extract contained nucleation-preventing agents. The limiting factors in stone formation could be those processes that affect crystal growth, because particles may become large enough to occlude the urinary tract, leading to stone formation.[28-32] 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.[25,33] Recurrent calcium stone formers excrete clusters of crystals in their urine, caused by aggregation, also named agglomeration, whereas urine from normal people contains mainly single crystals.[34-36] Again, percentage inhibition of crystals aggregation increased as the concentration of C-PC increased.

CONCLUSION

Antiurolithiatic activity of *S. platensis* is mediated possibly through inhibition of CaOx crystal formation and its effect on the urinary concentration of stone forming constituents and nephrolithiasis inducing factors and this study rationalizes its medicinal use in urolithiasis.

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Source of Support: Nil. **Conflicts of Interest:** None declared.