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Original Research

Cystone®, a well-known herbal formulation, inhibits struvite crystal growth formation in single diffusion gel growth technique

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Key Words
Biocrystallization; Cystone®; Renal calculi; Struvite stones

Abstract
Objective: The present study was aimed to evaluate the beneficial effect of Cystone® against struvite crystal growth in *in vitro* conditions.
Methods: Various concentrations of Cystone® was prepared in 1 M magnesium acetate solution and evaluated for crystal growth inhibition assay by a well-known method called single diffusion gel growth technique *in vitro*.
Results: Cystone®, a well-known polyherbal formulation, at 0.5, 1 and 2% concentrations showed significant and dose-dependent inhibition of struvite crystal growth formation in *in vitro* by reducing number, total mass and total volume of the struvite crystals formed and also caused fragmentation of grown struvite crystals in the gel matrix.
Conclusion: The results of the present study indicate, Cystone® significantly retards the formation of struvite stones and also brings about its fragmentation. This could be one of the probable mechanisms behind the beneficial effect offered by Cystone® in the clinical management of urolithiasis and urinary tract infections.

INTRODUCTION

Biocrystallization or biomineralization phenomena are the root cause of several diseases in humans; the most common example is urinary calculus [1]. A large number of people (up to 20% of the population worldwide) are suffering from urinary stone problem. The majority of stones are composed of oxalates, calcium salts, and phosphates. Among phosphates, magnesium ammonium phosphate hexahydrate (MAPH; $MgNH_4PO_4 \bullet 6H_2O$), known as struvite, is the predominant crystalline component [2]. It occurs as crystallites in urine and as a type of kidney stone; they are also called as triple phosphate stone or infectious stones or urease stones [3]. Worldwide, they compose 30% of all the kidney stones and are more common in women [3].

Struvite calculi is been referred as infection stones, they are associated with struvite urolithiasis [4]. Struvite crystals, if not treated may grow rapidly in to branched calculus in the renal pelvis and calyces and which leads to the blockage of the urinary tract and associated medical problems [5].

Currently, a long-term antibiotic treatment is advised in the case of infectious stones [6]. However, long-term systemic antibiotic therapy may lead to bacterial resistance and gastrointestinal adverse effects [7]. Interestingly, many plants such as *Moringa oleifera*, *Asparagus racemosus*, *Rotula aquatica*, *Mimosa pudica*, *Trigonella foenum graecum*, *Nigella sativa*, *Punica granatum* have been traditionally used to treat kidney stones and also they have been scientifically proved to be beneficial in the treatment of urolithiasis [8, 9].

In this context, Cystone® is a well-known polyherbal formulation manufactured by M/s The Himalaya Drug Company, Bangalore, India, based on the available

information in the ancient ayurvedic system of medicine, and has been used for many years to treat urinary calculi and urinary tract infection (UTI) [10, 11]. Considering the beneficial effects of cystone in treating the urinary caliculi and UTI, the present study was aimed to evaluate the beneficial effect of cystone against struvite stone formation in an *in vitro* model of single diffusion gel technique.

MATERIALS AND METHODS

Chemicals

Sodium meta-silicate, ammonium dihydrogen phosphate and magnesium acetate were purchased from Rankem Fine Chemical Limited (New Delhi, India); other chemicals and reagents used were of analytical grade and procured from Himedia Laboratories (Mumbai, India).

Cystone formulation

Cystone® is been approved by the Government of India's Drug Regulatory Authority (Department of Ayush, Ministry of Health and Family Welfare).

Each 5 millilitres of Cystone® syrup contains extracts of the following medicinal plants as major ingredients: Gokshura (*Tribulus terrestris*), 91 mg; Punarnava (*Boerhaavia diffusa*), 67 mg; Pashanabheda (*Saxifragaligulata*), 53 mg; Mustaka (*Cyperus rotundus*), 42 mg; Shatavari (*Asparagus racemosus*), 21 mg; Kulattha (*Dolichos biflorus*), 21 mg; Ushira (*Vetiveri azizanioides*), 21 mg; Karchura (*Curcuma zedoaria*), 14 mg; and powders of Saindhava, 50 mg; Suvarchika, 42.5 mg; Yavakshara, 5 mg; and Narasara, 2.5 mg.

Cystone® is available in the market from several decades. It has been tested for its quality and consistency in each and every step of manufacturing as per the accepted principles of GMP (good manufacturing practice) and GLP (good laboratory practice).

TLC fingerprinting of Cystone® syrup

Cystone® has been standardized with respect to various parameters which comply with the stringent quality check specification of the finished product. The product is standardized by physical and chemical parameters such as determination of total tannin and flavonoid contents, which represent the major chemical markers for standardization. Every batch of Cystone® is compared with the reference control in terms of various quality parameters and also unique TLC (thin layer chromatography) finger printing.

Preparation of sample solution

About 25 ml of Cystone® syrup was taken in a 250 ml flat bottom flask and extracted with 50 ml of dichloromethane on water bath for 30 min at 45°C. With the aid of a separating funnel the dichloromethane layer was separated and evaporated to dryness. The residue obtained was reconstituted with 2 ml of dichloromethane and 10 µl of the sample solution was spotted on pre-coated 20x20 cm thin layer silica plate (Silica Gel 60 F254 TLC Aluminium Sheets, EMD Merck, Darmstadt, Germany). The TLC chamber was pre-saturated with the mobile phase for 2 h and the sample spotted TLC plate was allowed to develop up to 15.5 cm in dichloromethane:methanol (95:5) solvent system. The developed TLC plate was exposed to saturated iodine vapours and visualized under uv-radiation at 254 and 366 nm wavelengths. The HPTLC finger print of cystone syrup has been given in Fig.1.

In vitro struvite crystal growth inhibition assay

Struvite crystal growth inhibition property of Cystone® was evaluated by single diffusion gel growth technique; this is an excellent in *vitro* model which exactly mimics the complex growth of urinary calculi in *in vivo*. In short, an aqueous solution of 0.5 M ammonium dihydrogen phosphate was mixed with sodium meta-silicate solution of specific gravity of 1.05 in appropriate quantity to set the pH value 7.2 and transferred to autoclaved glass test tubes for gel formation. After gel formation, 20 ml supernatant solution of pure 1 M magnesium acetate (control) and 20 ml supernatant solution of 1 M magnesium acetate solution containing various concentrations of Cystone® (0.5, 1 and 2%) were gently poured on the gels into the respective test tubes. This procedure was performed under aseptic conditions in laminar airflow hood to avoid microbial contamination. After pouring the supernatant solutions, the test tubes were closed with cotton tightly (air tight) and kept at room temperature and the formation of struvite crystals were observed for 21 days.

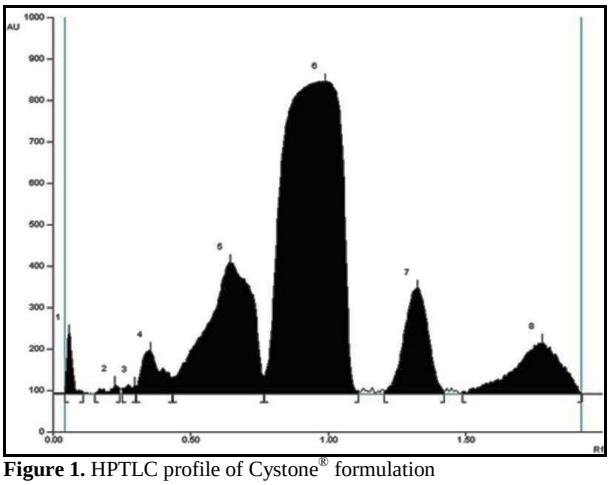

Figure 1. HPTLC profile of Cystone® formulation

The experiments were carried-out in triplicate and at the end of the study the crystals were gently removed from the gel-matrix and quickly washed with PBS (pH 7.2) solution. The total mass and volumes of crystals were measured accurately for all the samples including control [3].

Statistical analysis

The results were expressed as mean ± SEM and analyzed statistically by One Way ANOVA followed by Dunnett's multiple comparison test using GraphPad Prism version 4.00 for Windows (GraphPad Software, San Diego, CA, USA). The minimum level of significance was fixed at 95% confidence limit.

RESULTS

Present study was envisaged to study the effect of Cystone® on the formation of struvite crystals/stones by a well-known and commonly adopted technique called the single diffusion gel growth technique providing a simplified *in vitro* model of highly complex growth of urinary calculi *in vivo*, and it allows a practical observation crystal growth in its all stages [3]. In present study, Cystone® decreased the formation of struvite crystals in a concentration-dependent manner which was indicated by decrease in mass and volume of the crystals formed in the gel matrix containing the drug (Figs.2-4). However, control test tubes free from Cystone® showed rapid crystal growth, which is associated with increase in number, total weight and total volume of the crystals (Figs.2-4).

Furthermore, visual observation of the crystal size revealed the presence of large struvite crystals in the control test tubes. In contrast, 1% and 2% added test tubes have showed qualitatively smaller size of the struvite crystals compared to control, which could be due to the fragmentation of struvite crystals/stones in presence of Cystone® (Figs.5&6).

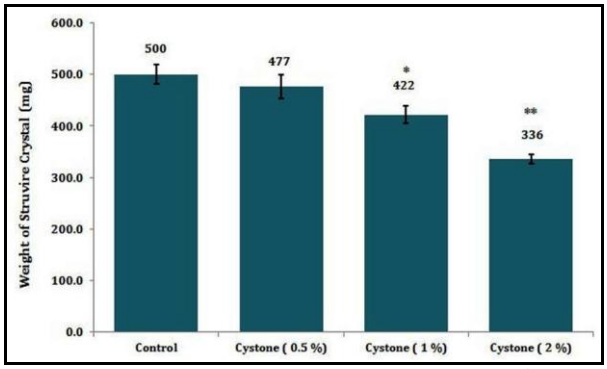

Figure 2. Concentration-dependent decrease in the mass of struvite crystals by Cystone®, after 21 days of crystal-growth in sillica hydro-gel medium. Values are expressed as mean of three trials (n = 3) ± SEM. *P < 0.05, **P < 0.01 compared to control.

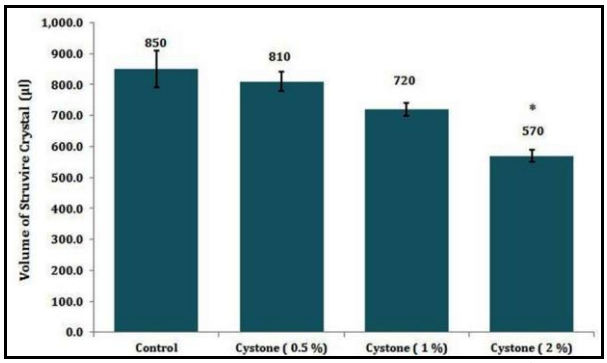

Figure 3. Concentration-dependent decrease in the volume of struvite crystals by Cystone®, after 21 days of crystal-growth in sillica hydro-gel medium. Values are expressed as mean of three trials (n = 3) values are expressed as mean ± SEM. *P < 0.05 compared to control.

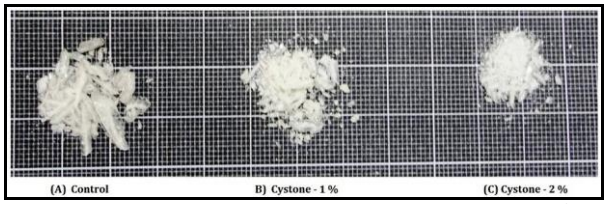

Figure 4. The photographs showing struvite crystals obtained from gel-medium; there is a significant change in volume and mass of crystals obtained from Cystone®-treated gel-medium **(B & C)** compared to control **(A)**.

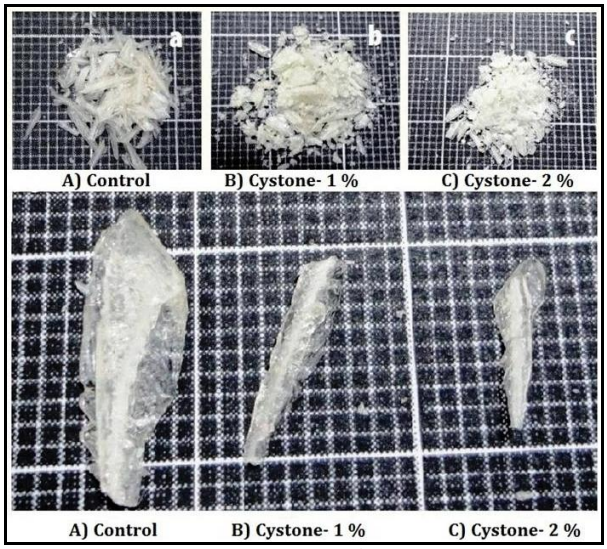

Figure 5. Photographs showing well-formed elongated dendritic struvite crystals obtained from gel-medium, the size of crystals obtained are reduced in Cystone®-treated gel-medium **(B & C)** compared to control **(A)**.

The findings of the present study suggest that Cystone® significantly retards the formation of struvite stones and also brings about its fragmentation.

DISCUSSION

The single diffusion gel growth technique provides a simplified *in vitro* model of highly complex growth of urinary calculi in *in vivo*, and it allows a practical observation crystal growth in its all stages [3].

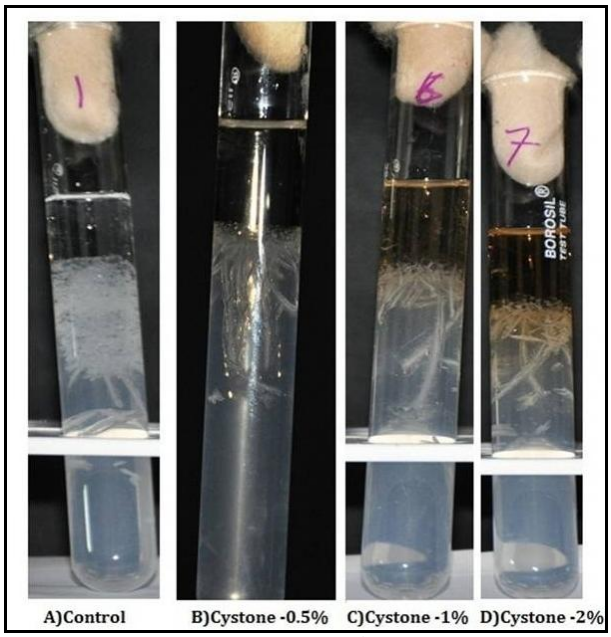

Figure 6. Test tubes showing a growth of struvite crystals (stones) in a gel matrix; there's a profuse growth of crystals in gel medium without drug **(A)**. Also, find concentration dependent decrease in struvite crystal formation in tubes containing Cystone® **(B, C and D)**.

In single diffusion gel growth technique, the gel acts as a 'three dimensional kettle' that supports the crystals and crystal growth without exerting major forces on it and thereby helps in the formation of urinary crystals with high structural perfection. The gel medium also mimics the synovial cartilage and other biological fluids and thus the crystals formed will exactly simulate the formation of crystals in human body [12].

The *in vitro* crystallization systems are widely used to study processes of crystal nucleation, growth and agglomeration, these mechanics would explore the pathophysiology of renal stone disease [9]. In literature several herbal drugs have been screened and proved to be effective in preventing the *in vitro* crystal growth [13-15].

Further, Cystone® has been proved to be very effective in preventing the supersaturation of lithogenic substances and additionally it possesses antioxidant activity [10, 11]. In addition, a study conducted by Vidyashankar *et al* [16], showed that Cystone® is also beneficial in preventing the adherence of uropathogenic *E.coli* to urinary epithelium and thereby acts as a antimicrobial agent to prevent the urogenital damage. By considering the literature reports on Cystone® related to antilithogenic, antioxidant and antimicrobial properties, In present study we have evaluated Cystone® for struvite crystal growth inhibition property in *in vitro*. The findings of the study revealed that Cystone® formulation could inhibit the nucleation and aggregation of magnesium ammonium phosphate hexahydrate struvite crystals in dose-dependent manner. Furthermore, the plants constituents such glycosaminoglycans, higher carboxylic acids such as citrates would chelate the divalent anions like as calcium and magnesium and thereby inhibit crystal growth and also facilitate their excretion in urine [12]. Interestingly, Cystone® showed significant and dose-dependent decrease in number, weight and volume of the struvite crystals by inhibiting the formation and facilitating the fragmentation.

Reduction in number, total mass and total volume of the struvite crystals formed; associated with fragmentation of grown struvite crystals in the gel matrix containing different concentrations of Cystone® demonstrated the concentration-dependent struvite crystal growth inhibition property of Cystone® in *in vitro* conditions.

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COMPETING INTERESTS

Author declares that there are no conflicts of interest.

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