An Aqueous Extract of the Leaves of *Ficus religiosa* Inhibits the Growth of Urinary Calcium Hydrogen Phosphate Dihydrate Crystals-An *In Vitro* Study

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Abstract

**Background:** *Ficus religiosa* L. (Lauraceae) has been shown to have numerous biological activities such as wound healing, antibacterial, anticonvulsant, antidiabetic and antiinflammatory. However, the effects of this plant on Calcium Hydrogen Phosphate Dihydrate (CHPD) urinary crystals remain obscure. The present study investigated the inhibitory action of an aqueous extract of the *Ficus religiosa* (F. religiosa) leaves on the growth of CHPD crystals.

**Methods:** CHPD crystals were grown as a urinary type using single diffusion gel technique in silica hydro-gels. An extract (50, 75 and 100%) of the leaves of *F. religiosa* was added over to CHPD crystals growth for 12 days to evaluate its inhibitory effect. Thermogravimetric (TGA) and powder X-ray diffraction analyses were performed to characterize the cultured CHPD crystals.

**Results:** A 75 and 100% aqueous extracts of the leaves of *F. religiosa* was significantly (P<0.05 and 0.01, respectively) reduced CHPD crystals growth and the inhibition rate was almost >50%. Whereas, a moderate inhibition (P<0.05) of CHPD crystal growth was found in 75% of aqueous leaf extracts of *F. religiosa*. However, 50% of aqueous leaf extracts of *F. religiosa* exhibit no inhibition on the CHPD crystal growth.

**Conclusion:** These results indicate that the higher concentration of the leaf extracts of *F. religiosa* is highly responsible for the inhibition of CHPD crystal growth. This study may suggest that *F. religiosa* can be used as therapeutic agent for the treatment of urinary calculi or their prevention.

**Keywords:** CHPD crystals; *Ficus religiosa* L; Liesegang ring; Powder X-ray diffraction; Thermogravimetry

Introduction

Urolithiasis, also called calculi or uroliths, is a condition which involves the process of stone formation in the kidney, bladder and/or urethra. Kidney stones are a general cause of blood in the urine and pain in the abdomen and flank, with a reported incidence about 12% in the general population [1]. There are numerous theories for the development of the urinary calculi [2,3]. The nucleation theory proposes that urinary stones originate from the crystals present in supersaturated urine. The crystal-inhibitor theory, furthermore, suggests that calculi form due to the absence or low concentrations of the host’s natural stone inhibitors. The development of urinary calculi can be simulated in the laboratory by growing crystals in a silica hydrogel medium. This growth of urinary crystals in silica hydrogel can be considered as a simplified *in vitro* model for the highly complex growth of urinary calculi in vivo.

The development of crystals in the gel is the simplest techniques under ambient environments which is appropriate for the crystal growth of compounds sparingly soluble and which decompose at low temperatures. India is expected to have urinary stones at about 12% of the population and out of that about 50% of cases encounter loss of one or both kidneys. It has been shown that upper as well as lower urinary tract stones arise habitually, but in India the incidence shows wide variation on the regional basis [4]. Therefore, it is essential to find for an alternative means such as medicinal plants or phytotherapy [5]. 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 [6].

*Ficus religiosa* L. (Lauraceae), is widely branched with long-tipped, leathery heart shaped leaves and purple fruits growing in pairs. It has mythological, religious and medicinal importance in Indian culture since ancient times [7,8]. This plant has long been used in traditional medicine for various disorders and its different parts have been used medicinally in various forms as well as in combination with other herbs. This plant has been shown to have numerous biological activities such as wound healing, antibacterial, anticonvulsant, antidiabetic, antiinflammatory and acetyl cholinesterase inhibitory activity [8-13]. The acetone extract of *F. religiosa* leaves has been shown to induce apoptosis in breast cancer cell lines [14]. Choudhari et al., have reported the antioxidant and cytotoxic activity of *F. religiosa* bark against cervical cancer cells [15]. A recent study has reported the anti-uler activity of the ethanolic extract of *F. religiosa* leaf [16]. Antitumor activity of biosynthesized silver nanoparticles using *F. religiosa* as a nanofactory in Dalton’s Ascitie Lymphoma (DAL) induced mice has recently been reported by [17]. In the present study,
for the first time we aimed at investigating the efficacy of *F. religiosa* on CHPD crystals growth *in vitro*.

**Materials and Methods**

**Growth of CHPD crystals**

The gel technique is found to be promising method to grow CHPD crystals as described by Joshi and Joshi, 2003 [18]. This technique provides much simplified method to understand the growth of urinary crystal *in vitro*. The formation of Liesegang rings was observed in the presents study. Glass test tubes were used as a crystallization apparatus and the single diffusion reaction technique was employed. One of the reactants, 1 M orthophosphoric acid, was mixed with sodium metasilicate solution having a specific gravity of 1.04g/cm³. After obtaining a clear transparent gel of optimum porosity, the supernatant solution of 1 M calcium chloride was gently poured onto the set gel in various test tubes. The experiments were repeated three times and each time three test tubes were used for the same supernatant solution. After pouring on each supernatant solution, the test tubes were capped with airtight stopples. The experiments were conducted at room temperature (~37°C). The growth parameters of CHPD crystals are given in table 1.

**Collection Ficus religiosa L. (Lauraceae) and its extract preparation**

Fresh leaves of *F. religiosa* (Figure 1A) were collected (2007 November) from the Botanical garden of Periyar Maniammai University, Vallam, Tamil Nadu, India. The plant material was verified by Dr. T Eevera, Professor Department of Biotechnology, Periyar Maniammai University. Collected plant specimen was systematically tagged, pressed, dried and mounted on herbarium sheets. Voucher specimens were deposited in the Department of Biotechnology, Periyar Maniammai University for future reference.

Leaves were shade-dried and then finely powdered. The powder (10 g) was extracted with 100 ml of distilled water using a Soxhlet apparatus (an apparatus which is used to remove or concentrate substances that may otherwise be very difficult to remove or concentrate if a manual process is used). The material thus obtained was filtered, and the resulting filtrate was concentrated to a dry mass by vacuum distillation; this was used for the current study.

**F. religiosa extract on the growth of CHPD crystals**

The putative activity of the plant extracts as inhibitors of CHPD crystal formation was investigated. The various concentrations (50, 75 and 100 g) of the plant extract were dissolved in the respective 50, 75 and 100 ml of distilled water to give 50, 75 and 100 % solution at the time of experiment. The prepared solutions were added to the formed CHPD gels and the results were noted. The experiments were repeated three times.

**Thermogravimetric analysis of CHPD crystals**

The Thermogravimetric Analysis (TGA) was performed on powdered samples by employing SDT Q600 V 8.3 Build 101 instruments set up. The thermogram was obtained by heating a sample from room temperature to 900°C, in an atmosphere of nitrogen, with heating rate of 15°C/min using a -Al₂O₃ as standard reference. The thermogram is shown in figure 1B. From this figure one can notice that hydrated calcium phosphate becomes anhydrous at 123.04°C, thereafter, at 552°C it turns into Calcium Pyrophosphate (Ca₃P₂O₇). The melting point of Ca₃P₂O₇ is 1230°C; therefore, it is expected to remain stable up to the end of the analysis, that is, 900°C. The following chemical reactions are expected to occur during the dehydration and decomposition stages.

**Powder X-ray diffraction analysis of CHPD crystals**

Powder X-ray diffraction pattern was recorded on Bruker advance diffract meter within the range 20 of 10 to 80°C. The elemental composition of the specimen was determined using an elemental analyzer with energy dispersive X-Ray Fluorescence system (XRF). The surface morphology of the samples was evaluated by Scanning Electron Microscopy (SEM). Thermal analyses were performed using SDT Q600 V8.3 build 101 instrument. FTIR spectra of the grown crystals were recorded using Perkin Elmer, Spectrum Rx1 detector and KBr beam splitter.
Results

The first Liesegang ring was observed within 12 min of pouring the solution. In total, 18 Liesegang rings were observed over time. The elongated platelet shape CHPD crystals grew within the rings. In addition, platelets originated from a single point, that is, star shaped crystals were observed. Figure 2 shows the schematic diagram of the growth of crystals at 12th day (Figure 2A), an enlarged picture (Figure 2B) and the morphology (Figure 2C) of harvested CHPD crystals. The largest crystal was 18 mm in length. The crystals were characterized by Fourier transform infrared spectroscopy, thermogravimetric analysis, scanning electron microscopy and powder x-ray diffraction methods, and confirmed to be CHPD.

The morphological characters of harvested CHPD crystals show (Figure 2C) that the crystals are in needle or star shaped. The crystal length ranges about 2.3-3 cm, the crystal breadth is 1-2 mm and their thickness was about 1 mm (Table 2). The thermogram was obtained by heating a sample from room temperature to 900 °C in an atmosphere of nitrogen with heating rate of 15 °C/min, using α-Al2O3 as standard reference. A picture of thermogram and the thermal composition of CHPD crystals are shown in figure 1B and table 3, respectively.

The X-ray powder diffraction of the cultured CHPD crystals (Table 4) was matched with the organic database using computer and the results were consistent with CHPD X-ray powder diffraction gram (Figure 3A) produced during the x-ray scans. The peaks positions represent where the x-ray beam has been diffracted by the CHPD crystals. The set of d spacing (the distance between the adjacent planes of atoms), which represents the unique “finger prints” of the crystals can easily calculated from the 2-theta (2θ) values. The uses of the degrees 2-theta in depicting x-ray powder diffraction scans is a matter convention and can easily be related peak to the JC-PDF (Joint Committee for Powder x-ray Diffraction File). The comparing the measured diffraction grams with JC-PDF has identified constituents present in the CHPD crystals.

The major and interesting finding of this study is the extract of the leaves of F. religiosa inhibited the formation of CHPD crystals (Figure 3B). The results of the decreased optical density with the increase in concentration of the extract of F. religiosa indicating that

Discussion

Ficus religiosa is reported to have several therapeutic uses in folk medicine. Their leaf juice has been used for the treatment of asthma, cough, sexual disorders, diarrhea, hematuria, ear-ache and toothache, migraine, eye troubles, gastric problems and scabies; leaf decoction has been used as an analgesic for toothache; fruits for the treatment of asthma, other respiratory disorders and scabies; stem bark is used in gonorrhea, bleeding, paralysis, diabetes, diarrhea, bone fracture, antiseptic, astringent and antidote [19]. In Ayurveda, it is claimed that F. religiosa possesses anticonvulsant activity [20]. Acetyl cholinesterase inhibitory and antianxiety activities of this plant were also studied [13,21]. However, there is no information about their inhibitory effects on kidney stone and/or CHPD, oxalate formation. The main findings of the present study show that an extract of the leaves of F. religiosa inhibited the formation of CHPD crystals in a concentration dependent manner; less and smaller particles were formed with increasing concentrations of the extract. 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 [22]. Chaudhary et al., have suggested that the limiting factors in stone formation may affect the crystal growth, because particles may become large enough to occlude the urinary tract, leading to stone formation [23]. The herb extracts may contain substances that inhibit the growth of CHPD crystals.

It has been reported that oxalate plays an important role in stone formation and has about 15-fold greater effect than urinary calcium [24,25]. In the present study, CHPD is formed competitively in an in vitro gel system. Joshi et al., have reported the inhibitory effects of litholytic medicinal plants Tribulus terrestris L. (Zygodiphyllaceae) and Bergenia ligulata L. (Saxifragaceae) on the growth of CHPD crystals [26]. They have described that calcium chloride containing supernatant solution produced Liesegang rings in the gel and needle, platelet and star-shape CHPD crystals which are grown within the rings. The calcium chloride in the supernatant solutions was modified the diffusion process and thus the periodic precipitation and the number of Liesegang rings reduced by the addition of aqueous extracts of T. terrestris and B. ligulata [26]. They have also noted the maximum length of the crystals reduced due to inhibition produced by the addition of aqueous extracts of B. ligulata and T. terrestris. Several lines of evidence indicated that there are various compounds exhibiting inhibitory actions on the growth of urinary stones and crystals, such as tartrates are good inhibitors of stones in natural and artificial urine [27]. In the present study, an extract of F. religiosa was used to study the growth behavior of CHPD crystals. F. religiosa inhibits the growth of CHPD crystals. This can be verified from the results of the formation of CHPD crystals.

Numerous inhibitors have been identified in urine for the calcium phosphate and calcium oxalate crystal systems. Magnesium, citrate, pyrophosphate and nephrocalcin are the inhibitors in the calcium phosphate crystal system [28]. The in vitro formation of urinary stones and the generation of spherulites of calcium phosphate in gels, as well as the overgrowth with calcium oxalate using a new flow model of crystallization have been studied by Achilles et al. [29]. Ethanolic extract of T. terrestris fruits were found to exhibit protection against uroliths induced by glass bead implantation in rats [30]. In another study, an administration of a drug containing T. terrestris to sodium glycolate fed rats produced a significant decrease in urinary oxalate excretion and a significant increase in urinary glyoxylate excretion [31]. Recently various herbal plants such as Flos carthami, Costus igneus, Tribulus terrestris and Scoparia dulcis have successfully proved as prophylactic and curative medicine for urolithiasis [32-35]. Another recent in vitro study showed that leaf extracts of I. eriocarpa possesses potent antiurolithic activity [36]. So remove also, the results of present study corroborate findings with the results of the above investigations.

Conclusion

The findings of the present investigation shed light on the inhibitory processes occurring in an extract of the leaves of F. religiosa on the growth of CHPD crystals. Despite, the process of stone formation in the human body is fairly complicated the present study provided basic information, under laboratory conditions, which led us to identify new inhibiting solutions of stone growth. Further in vivo investiga-

Figure 3A: Powder X-ray diffractogram of CHPD crystals.

Figure 3B: Effect of an extract of the leaves of F. religiosa on CHPD crystal growth: a) (CHPD crystal), b) (CHPD crystal treated with 50% F. religiosa extract), c) (CHPD crystal treated with 75% F. religiosa extract) and d) (CHPD crystal treated with 100% F. religiosa extract).
tions will be done in the future to clarify the molecular mechanism of the inhibitory action of F. religiosa on the growth of CHPD crystals. The results of this study may suggest that F. religiosa can be used as therapeutic agent for the treatment of urinary calculi or their prevention.

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References


