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Research Article
International Journal of Pharmacy and Engineering (IJPE)
ISSN 2320-849X

Fractions from Coconut water (*Cocos nucifera*) influencing *in vitro* calcium oxalate crystal growth

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Abstract

Urolithiasis, the formation of calculi in urinary tract, has been a major problem for mankind since ages. Although the surgical techniques such as Extracorporeal Shock Wave Lithotripsy (ESWL) and Percutaneous Nephrolithotomy (PN) revolutionized the management of urolithiasis yet these approaches have some limitations. So, it is worthwhile to look for an alternative management of urolithiasis, hence the significance of phytotherapy. The current study evaluated the *in vitro* potential of antilithiatic fractions from coconut water towards calcium oxalate growth. The coconut water was also evaluated for *in vitro* inhibitory and stimulatory potential of mineralization and demineralization reactions, respectively. Inhibitory activity of coconut water fractions was tested by observing a decrease in growth of calcium oxalate seed crystals in the presence of a test sample. The active fraction of coconut water was partially purified by dialysis, ion exchange chromatography and molecular sieve chromatography. Coconut water was found to inhibit *in vitro* homogeneous precipitation of calcium phosphate crystals from supersaturated solution (initial mineralization). Moreover, it stimulated dissolution of preformed calcium

phosphate crystals (demineralization). Fractions obtained from coconut water on anion exchange inhibited seeded calcium oxalate growth by 38.77% while that from molecular sieve chromatography caused 70.1% inhibition of calcium oxalate growth.

Key words: phytotherapy; urolithiasis; mineralization; demineralization;nephrolithiasis

Introduction

Urolithiasis has plagued mankind from antiquity and its persistent prevalence continues to pose a universal health problem having considerable socio-economical implication. The problem gets further compounded by high recurrent frequency of urinary stone disease. Epidemiological studies have shown that the incidence of disease varies among different countries. Kidney stones are among the most common painful disorders of urinary tract. Kidney stones disease affects upto to 5% of the population, with a lifetime risk of passing a kidney stone of about 8-10% [1]. Calcium oxalate (CaOx) is the primary constituent of the majority of stones formed in the urinary system of patients with urolithiasis [2]. In addition to calcium oxalate, urinary stones have been found to contain calcium phosphate, uric acid and magnesium ammonium phosphate.

Although, in recent years, development of modern techniques such as extracorporeal short wave lithotripsy (ESWL), percutaneous nephrolithotomy (PCNL) have revolutionized the surgical management of the problem, however, their application is not without side effects and kidney damage during ESWL is clinically significant problem [3]. Therefore alternative options are now employed for the management of urolithiasis. A large number of Indian medicinal plants are being used routinely by practitioners of Ayurvedic system of medicine in the treatment of urolithiasis [4, 5]. Urinary stone disease is comparatively low in South India [6] where coconut water is an essential dietary ingredient. Hence, the present study was undertaken to investigate the effect of coconut water on *in vitro* initial mineralization and demineralization reactions of calcium phosphate crystals. Further coconut water fractions were tested for their effect on seeded calcium oxalate crystal growth.

Results and discussion

The effect of coconut water on calcium phosphate crystallization from supersaturated solution (initial mineralization) and on the release of Ca^{2+} or HPO_4^{2-} ions (demineralization) from preformed mineral phase are presented in Tables 1 & 2. It was found that 10% coconut water inhibited initial mineralization of calcium phosphate in a dose dependent manner [Table 1].

Further, an increase in the release of Ca^{2+} and HPO_4^{2-} ions from the preformed mineral phase was observed in the presence of coconut water [Table 2]. Coconut water was subjected to purification of active fraction(s) by dialysis using anion-exchange & molecular sieve column chromatography. Figure 1 shows the elution profile of anion-exchange chromatography of >10 kDa dialysant of crude coconut water. The fractions under the peak obtained after anion-exchange chromatography were pooled and checked for their inhibitory activity. Fractions pooled between time intervals 132-141 min. showed maximum inhibition, 38.77% with respect to control towards CaOx crystal growth assay system at 1200 seconds (Figure 2).

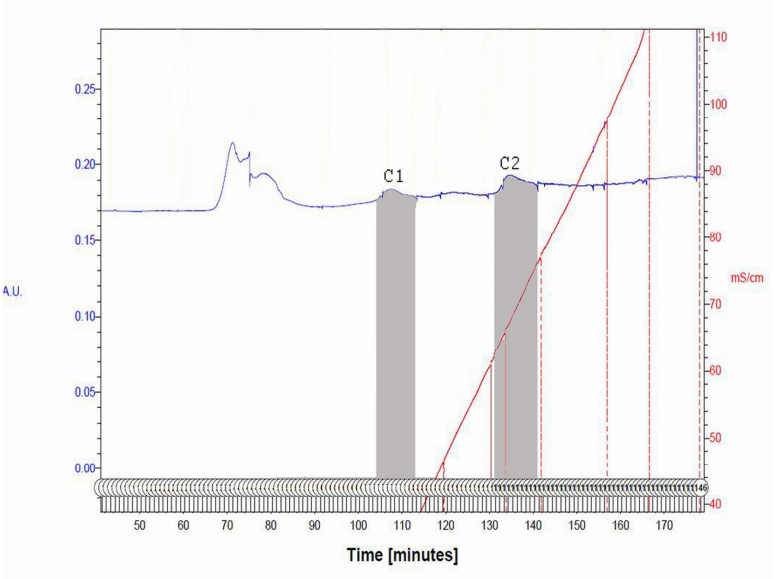

Figure 1. Elution profile of anion-exchange chromatography of >10 kDa dialysant of crude coconut water

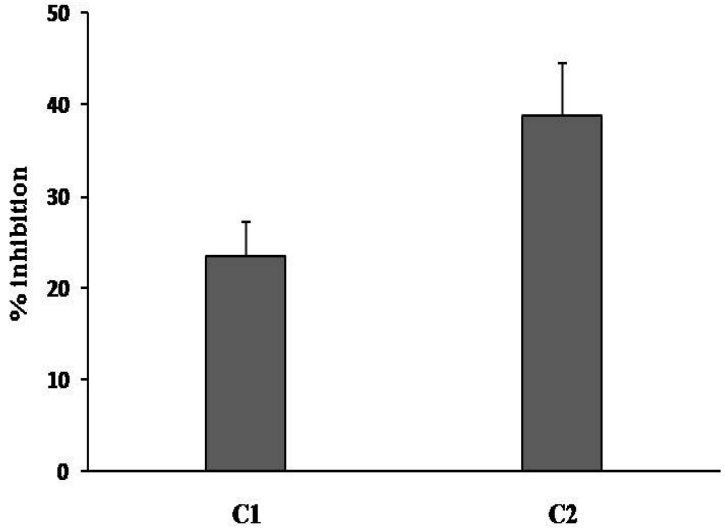

Figure 2. Inhibitory potency towards calcium oxalate growth

Since C1 (104-113 min.) and C2 (132-141min.) fractions showed inhibition towards calcium oxalate assay system (Figure 2), these fractions were pooled, lyophilized and subjected to molecular sieve chromatography (Figure 3). Fractions obtained from molecular sieve chromatography were checked for their inhibitory potency towards *in vitro* calcium oxalate assay system. Percent inhibition of calcium oxalate crystal growth by various fractions obtained after molecular sieve chromatography as depicted in (Figure 4) reveals that peak eluting at 900-1050 min. (K5) showed the highest inhibitory activity towards *in vitro* calcium oxalate assay system. The inhibition by the fraction of this peak was 70.1% at the interval of 1200 sec of calcium oxalate growth assay (Figure 4).

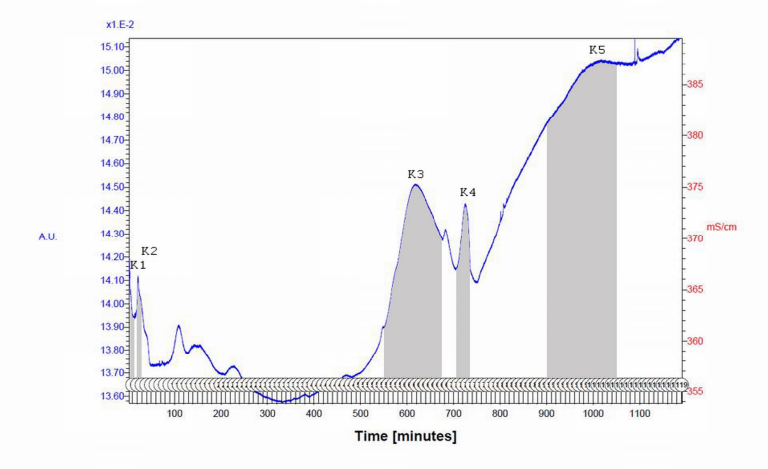

Figure 3. molecular sieve chromatography

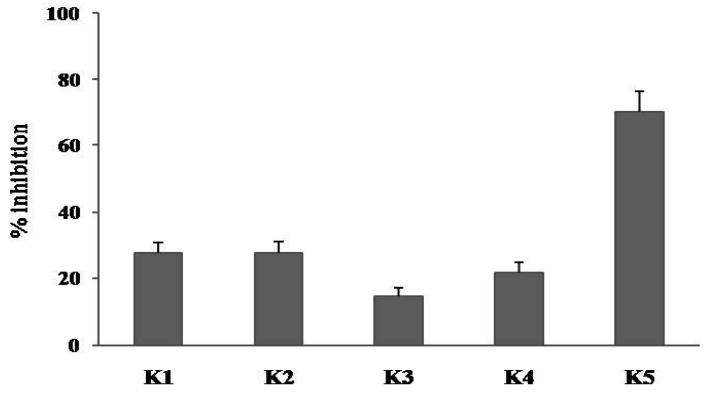

Figure 4. Percent inhibition of calcium oxalate crystal growth

Formation of urinary stones depends on a delicate balance between thermodynamic and kinetic factors regulating supersaturation [7]. Only when urine is supersaturated, calcium salts crystallize, grow and aggregate to form stones[8] . Several natural products [5, 9, 10] have been shown to inhibit crystallization and growth of calcium salts both *in vitro* and *in vivo*. The present study demonstrated that coconut water inhibits calcium phosphate precipitation and stimulates dissolution of such crystals. Further it inhibits calcium oxalate crystal growth. Known constituents of coconut water such as sugars, vitamins, minerals, free amino acids and growth promoting factors are unlikely to potent candidates for inhibitory activity which has been found to be associated with a fraction having molecular weight > 10kDa.

Conclusion

The results presented herein suggest that coconut water inhibits *in vitro* crystallization of calcium phosphate mineralization and seeded crystal growth of calcium oxalate (two major constituents of urinary stones). Further coconut water also stimulates dissolution of calcium phosphate crystals. The present study tends to explain a lower incidence of urolithiasis in South India where coconut is essential dietary ingredient. Although exact nature of active principle could not be ascertained in the present study, yet it suggests molecular weight of the active component to be more than 10 kDa.

Experimental

Collection of coconut water

Fresh coconut fruits *(Cocos nucifera L.)* were procured from the local market and broken carefully to obtain endosperm. Coconut water was centrifuged at 3000 rpm for 30 min at 4°C in cold centrifuge and the supernatant was used for the study.

Dialysis of the samples:

The dialysis tubes (molecular weight cut off 10 kDa) were cut into 3 inch long pieces before use. A known amount of coconut water sample was transferred into each of the dialysis tube and the dialysis was conducted for 72 h against phosphate buffered saline with constant stirring at 4°C in a cold room with 24 hourly change in buffer. At the end, dialysis tubes were removed and various dialysates and dialysants recovered at different intervals were pooled. All pooled dialysates and dialysants were concentrated to a known volume using lyophilizer.

Fractions from coconut water

The dialysant obtained from above was centrifuged at 10,000 rpm for 15 min at 4°C to remove insoluble materials and filtered through Whatman No. 1 filter paper. The Macro prep® 25 Q strong anion exchanger, after removal of ethanol, was pre-equilibrated with 20 mM Tris buffer (pH 7.4) containing 0.1 M NaCl at and packed into (20 x 1.5 cm) column. The column was eluted with a linear gradient of 0.1-1.0 M sodium chloride at a rate of 1ml/min. The fractions having inhibitory potency towards calcium oxalate growth (figure 2) were pooled, dialyzed against 50 mM Tris–Cl containing 50 mM NaCl and further fractionated on a Bio gel ® P-100 gel equilibrated and eluted with the 20 mM Tris buffer (pH 7.4) at a flow rate of 0.1 ml/min. The fractions under each peak were pooled and checked for their *in vitro* inhibitory potency towards calcium oxalate crystal growth.

2.5 *In vitro* calcium phosphate (CaP) assay:

Homogeneous mineralization system was used to study the extent of *in vitro* mineral phase formation in the absence of any matrix [11]**.** This *in vitro* homogeneous assay system was modified by replacing 17.5 mM barbital buffer with 0.1 M Tris buffer (pH 7.4). The assay system consisting of $CaCl_2$ (5 mM), K_2HPO_4 (5 mM), Tris buffer (0.1 M, pH 7.4) and NaCl (105mM) in a final volume of 5ml was incubated for 30 min. at 37°C in the absence (control) or presence of coconut water (test). The precipitates obtained were dissolved in 5ml of 0.1 N HCl and their $Ca^{2+}$and HPO_4^{2-} ions were estimated by the methods of Trinder (1960) and Gomori (1941), respectively. The Percent inhibition of mineral phase in the presence of test sample was calculated as: Percent Inhibition = [(C - T)/C] x 100, where C and T are the concentration of Ca^{2+} or HPO_4^{2-} ions in the control and test systems, respectively. For demineralization reaction, initial calcium phosphate precipitates obtained as above in absence of coconut water were resuspended in fresh medium consisting of 0.1 M Tris HCl buffer (pH 7.4) and NaCl (105 mM) in a final volume of 5ml in absence (control) and presence of varying volumes of coconut water (test). The residual precipitates were dissolved in 0.1 N HCl and their $Ca^{2+}$and HPO_4^{2-} concentration were determined as above. Their Percent stimulation of demineralization was calculated from the equation: Percent stimulation = [(C - T)/C] x 100 where T is the concentration of Ca^{2+} or HPO_4^{2-} ions of the residual precipitates in the test and C is their concentration in the precipitates in control.

2.4 Crystal Growth Inhibition:

In vitro inhibitory activity against calcium oxalate crystal growth was measured using the seeded, solution-depletion assay described previously [12, 13]. Briefly, an aqueous solution of 10 mM Tris-HCl containing 90 mM NaCI was adjusted to pH 7.2 with 4 N HCl. Stone slurry (1.5 mg/mL) was prepared in 50 mM sodium acetate buffer (pH 5.7). Calcium oxalate crystal seeds (from FTIR identified clinical kidney stones) were added to a solution containing 1 mM calcium chloride ($CaCl_2$) and 1 mM sodium oxalate ($Na_2C_2O_4$). The reaction of $CaCl_2$ and $Na_2C_2O_4$ with crystal seed would lead to deposition of calcium oxalate on the crystal surfaces, thereby decreasing free oxalate that is detectable at wavelength of 214 nm. When a sample is added into this solution, depletion of free oxalate ions will decrease if the sample inhibits CaOx crystal growth. Rate of reduction of free oxalate was calculated using the baseline value and the value after 30-s incubation with or without sample. The relative inhibitory activity was calculated as follows: % Relative inhibitory activity = [(C - S)/C] x 100, where C is the rate of reduction of free oxalate without any sample and S is the rate of reduction of free oxalate with a test sample.

Table 1. Effect of Coconut water (10%) on initial mineral phase formation.

Volume of Coconut water	% Inhibition of ions precipitated	
(ml)	Ca^{2+}	HPO_4^{2-}
0.2	0	16.17 ± 2.74
0.4	10.18 ± 1.78	24.26 ± 3.69
0.6	34.70 ± 3.05	27.57 ± 4.58
0.8	74.60 ± 7.89	54.04 ± 5.76
1.0	94.11 ± 8.24	91.9 ± 6.43

Table 2. Effect of coconut water (10%) on the demineralization of pre-formed mineral phase

Amount of sample used (ml of 10% coconut water)	% Stimulation of ions released (demineralization)	
(ml)	Ca^{2+}	HPO_4^{2-}
0.2	0	16.79 ± 2.1
0.4	5.82 ± 1.32	19.53 ± 3.54
0.6	13.67 ± 2.54	25.39 ± 4.58
0.8	93.31 ± 6.79	75.70 ± 5.76
1.0	98.0 ± 7.42	76.95 ± 6.43

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