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Original Research Article

Evaluation of hydro-alcoholic extract of *Dolichos biflorus* seeds on inhibition of calcium oxalate crystallization

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ABSTRACT

Objective: To evaluate the effect of an extract obtained from *Dolichos biflorus* (Fabaceae) on calcium oxalate crystallization *in vitro*.

Materials and methods: A hydro-methanolic extract of (30:70, v/v) of *D. biflorus* seeds at different concentrations (1–10 mg/ml) was subjected to *in vitro* anticrystallization activity using a synthetic urine system. The results were compared with a parallel study conducted with the herbal medicinal product cystone under identical dosage conditions. The nucleation and aggregation of calcium oxalate crystals were measured using spectrophotometric methods and crystals generated in the urine were also analyzed by light microscopy. Statistical differences and percent inhibitions were calculated using GraphPad prism 5 software.

Results: The seed extract was significantly more effective than cystone at inhibiting the nucleation, as well as the aggregation of calcium oxalate monohydrate crystals in a dose-dependent manner. These results were also confirmed by the microscopic analysis.

Conclusion: The results showed that the selected herb, *D. biflorus* has excellent anticrystallization activity and therefore, might be beneficial if used in formulating a strategy for the dissolution and thereby prevention of urinary stones.

1. Introduction

The worldwide incidence of urolithiasis is increasing and calcium oxalate (CaC_2O_4) is the primary constituent of the majority of stones formed in the urinary system of patients with urolithiasis (Trinchieri, 2008). Studies report that the prevalence rate varies from 2–13% in developed countries to 0.5–1% in developing countries (Trinchieri, 2008). Areas with higher incidence of kidney stones are Scandinavian countries, Mediterranean countries, British Isles, Northern Australia, Central Europe, portions of the Malayan Peninsula, China, Pakistan and northern India (Trinchieri, 2008). India has higher incidence of urinary calculi, particularly in Gujarat, Rajasthan, Punjab and Madhya Pradesh (Shah, 2003). CaC_2O_4 urolithiasis accounts for approximately 75% of urinary stone disease in the United States (Trinchieri, 2008). Many studies from India have also documented that CaC_2O_4 forms the major

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constituent of renal calculi disease (Butterweck and Khan, 2009). There are two different types of CaC_2O_4 stones, CaC_2O_4 monohydrate (COM) or Whewellite, and CaC_2O_4 dihydrate (COD) or Weddellite. COM is thermodynamically the most stable form and is observed more frequently in clinical states and has a greater affinity for renal tubular cells (Butterweck and Khan, 2009). Urolithiasis is a complex process that results from the succession of several physicochemical events including supersaturation, nucleation, growth, aggregation and retention within the renal tubules. The supersaturation of urine results in nucleation which is defined as the formation of the solid crystal phase in a solution (Finlayson and Reid, 1978). This is followed by the crystal growth, which is the driving force for the particle formation and thus for stone formation (Masao, 2008). Then crystal aggregation occurs which is promoted by viscous binding *i.e.*, foreign crystalline compounds with multiple binding sites, such as abnormally self-aggregated macromolecules attach to the crystal surfaces (Masao, 2008). And finally aggregated crystals are retained in the urinary tract which can be caused by the association of crystals with the epithelial cells lining the renal tubules. Thus, one approach to prevent stone formation would be to stop crystal retention by reducing crystal nucleation and aggregation.

Significant development has been achieved in the management of urolithiasis following the introduction of several modern techniques, including extracorporeal lithotripsy and nephrolithotomy. However, stone fragments that are retained after treatment may serve as nidi for the formation of new stones. Therefore, effective therapies to prevent stone recurrence are still required. Unfortunately, despite considerable progress in medical therapy, there is no satisfactory drug to treat kidney stones. Thus, it appears useful to look for new prophylactic measures to be used either alone or in combination with already existing methods. In this regard, traditional herbal remedies can be a potent source of new antiurolithiatic remedies, because their extracts and compounds isolated from them have demonstrated varied biological activity.

A number of plants have been used in India and elsewhere which claim to prevent CaC_2O_4 crystallization (Butterweck and Khan, 2009). *Herniaria hirsuta L.* has been reported to promote the nucleation of CaC_2O_4 crystals, increasing their number but decreasing their size (Atmani and Khan, 2000). *In vitro* studies in which CaC_2O_4 precipitation was induced by addition of 0.1 M sodium oxalate to unfiltered urine samples from Wistar rats and normal humans in the absence and presence of *Phyllanthus niruri* extract (0.25 mg/mL), suggested that this extract may interfere with early stages of stone formation (Barros et al., 2003).

Dolichos biflorus is a genus of the family Fabaceae, a leguminous edible pulse crop of the subtropics, commonly known as Kulthi in the Indian Systems of Medicine and is a branched or trailing annual plant, with small trifoliate leaves and very wide climbing, slender stems. The seeds of *D. biflorus* have been reported to show antihepatotoxic, anticancer, antineoplastic, antinephrotoxic, antiatherosclerosis (Gangarao et al., 2011), antihyperlipidemic (Muthu et al., 2005), free radical scavenging activity and antioxidant properties (Hazra et al., 2009). In an earlier study, Garimella et al. (2001) demonstrated that an extract prepared from the seeds of *D. biflorus* could inhibit the precipitation of calcium and phosphate *in vitro*. In our previous *in vivo* studies, an extract from the seeds of *D. biflorus* demonstrated a significant nephroprotective effect under *in vivo* conditions (Saha and Verma, 2015) and protective effect in an ethylene glycol-induced rat model of nephrolithiasis (Saha and Verma, 2014).

Cystone is a marketed composite herbomineral formulation specifically developed for the management of urolithiasis or renal calculi. This formulation has been approved by regulatory authorities in India as an Ayurvedic formulation and has been available for clinical practice for the past 60 years (Mohanty et al., 2010). Cystone has been investigated for its safety and efficacy in urolithiasis under *in vivo* (Rao and Rao, 1998) as well as in double-blind, placebo-controlled studies (Kumaran and Patki, 2011). Therefore, the authors then compared our plant extract with cystone at the same dose levels to determine the potency of our extract.

Several scientific papers have already been published on anticrystallization effects from medicinal plants. *D. biflorus* is one of such plants that has been advocated as a traditional medicine for kidney stones. However, no reports of the preventive effect of *D. biflorus* on CaC_2O_4 crystallization have been published to date. Therefore, in the present study the *in vitro* anticrystallization activity of an extract of *D. biflorus* seeds has been investigated.

2. Materials and methods

2.1. Plant material

The *D. biflorus* seeds were collected in May 2011 and were taxonomically identified and authenticated by Dr Hitesh A. Solanki, Department of Botany, Gujarat University, India. A voucher specimen was deposited in the herbarium for future reference. The seeds were thoroughly cleaned, shade dried and coarsely powdered and a hydro-alcoholic extract was prepared according to the World Health Organization (WHO) protocol CG-06. Briefly, 5 g of seed powder and 100 mL of methanol: water (70:30, *v/v*) were mixed and stirred on a magnetic stirrer and then filtered twice with Whatman filter paper no 1. After evaporation of the solvent, the crude extract was dried under vacuum and stored in air tight containers at 4 °C until use. The yield of dry hydro-methanolic extract was 11.5% (*w/w*). The dried extract was dissolved in distilled water and used for further study in a range of concentrations as outlined in Section 2.4.

2.2. Chemicals and apparatus

Cystone (Batch No.: A029156 B) was obtained from Himalaya Drug Company, Bangalore, India. Quercetin dihydrate (Batch No.: T-836708) was purchased from Sisco Research Laboratories, (Mumbai, India). All the other chemicals used in the study were of analytical grade and procured from Hi Media Laboratories Pvt. Ltd., Mumbai, India and precoated silica gel 60 F_{254} TLC aluminum plates were purchased from Merck, Mumbai, India.