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In vitro urolithiasis models: An evaluation of prophylactic management against kidney stones

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Abstract
Urolithiasis is a global health problem with high recurrence rate. Different *in vivo* and *in vitro* models have been successfully used to evaluate the antiurolithiatic potential of medicinal plants. *In vitro* models provide the study of renal stone formation and *in vivo* models declare pathological effects of urolithiasis. Thus, *in vitro* models are significantly and effectively used to evaluate prophylactic management and *in vivo* gives the direction towards urolithiasis treatment. This paper describes the advantages, limitations and applications of both model specially, the role of *in vitro* studies in the evaluation of prophylactic management.

Keywords: Antiurolithiatic, *In vitro*, *In vivo*, urolithiasis, drug discovery, prophylactic management

Introduction
Urolithiasis is commonly referred as stone formation in any part of the urinary tract such as kidneys, ureters, urinary bladder and urethra. It is one of oldest, most frequent and highly recurrent disease and was initially found in the tombs of Egyptian mummies dating back to 4000 BC [1]. Epidemiological studies revealed that urolithiasis is more common in men than in women and is more prevalent between the ages of 20 to 40 in both sexes [2]. Uroliths are generally composed of calcium as calcium oxalate monohydrate and calcium hydrogen phosphate dihydrate (75-90%), magnesium as ammonium magnesium phosphate hexahydrate (10-15%), uric acid and urates (3-10%); and 0.5-1% is composed of cystine, hippuric acid, L-tyrosine and xanthine. Calcium containing uroliths are known as brushite, whewellite, weddellite, whitlockite and carbonate apatite. Struvite and newberyite are magnesium containing where as ammonium acid urate, mono sodium urate monohydrate, uric acid anhydrous, uric acid mono and dihydrate are commonly existing urate stones [1, 3]. Medicinal plants are considered as a rich source of therapeutic agents due to the belief and observations regarding their traditional use for the prevention of various ailments. Various research findings and data from different part of the globe are contributing and helping the scientific community in evaluating and establishing the pharmacological activities of these plants.
Different *in vitro* and *in vivo* (animal models) studies are used for determining various mechanism profiles of testing extract or compounds against different pathophsiological conditions and also for urolithiasis. These models best describe both prophylactic and curative action of the test sample. *In vitro* studies provide preliminary examination before conducting *in vivo* studies. These studies also provide smaller set-ups using a little test substance, allowing low costs and high number of replicates. Thus, providing motivation to minimize drug costs through economical testing procedures and gives more direct assess toward extract or compound performance than do conventional *in vivo* studies and therefore provide important insights into fundamental mechanisms for biological effects. It also avoids or decreases the need for laboratory personnel experienced in animal handling and there is no need to submit permission by the institutional animal ethics committee [4].
Urolithiasis is a multistep bio-chemical process with high recurrence rate. After urolithiasis treatment, there is 50% chance of stone formation within 7 years if left untreated. Therefore, prophylactic management is of great importance and advisable, especially in such individual subjects [5]. Crystallogenesis is the first and essential step in stone formation which is based on three steps nucleation, growth and aggregation.

In vitro antiurolithiatic studies assure prophylactic management through evaluation of nucleation, growth and aggregation inhibition of developed urinary crystals. In most of the studies nucleation inhibition suggested decrease in the bioavailability of minerals promoting crystal formation. It has been reported that masking of crystal binding sites in renal epithelial cells and other type of crystals can inhibit aggregation leading to the development of unhealthy crystals. The formation of defected crystals and conversion into a modulated form and thus does not allow to contribute pathogenesis or development of any pathophysiological conditions.These approaches will be discuss later on with the special reference of citric, phytic and tartaric acid.

Besides these advantages there are some limitations also as, *in vitro* tests just provides early phase testing strategy and cannot provide mechanistic approach to further explain *in vivo* findings. These models are also unable to provide and represent exact physiological conditions such as body temperature of animals, the blood electrolyte concentrations of species, the extracellular matrix or the extent of cell contacts [6]. Similarly, these models only relate to one aspect of urolithiasis e.g., crystallization and artificially simulated different phases of crystallization (nucleation, aggregation and growth) used to demonstrate pathological stones and some important data about the mechanism of urolithiasis [2, 7]. *In vitro* urolithiasis models can evaluate the ability of the test extract or plant compound to dissolve preformed crystals. The crystals are allowed to grow in a suitable medium for a period of time then inhibition or promotion of urinary crystal aggregation and growth usually being observed [4]. Therefore, these models cannot provide all aspects of the pathogenesis, including the anatomic and physiological role of kidneys as animal models do. So, these models are commonly applied for prophylactic management to prevent stone recurrence by decreasing the risk of crystal formation [8]. Different *in vitro* models are commonly used to study the urolithiasis and antiurolithiatic effects. Titrimetric estimation measures undissolved calcium oxalate by using $KMnO_4$ [9]. Colorimetric estimation of calcium phosphate usually observed by a colorimeter at 600-750nm. The presence of undissolved crystals in control and treated conditions provide better idea about inhibition and / or promotion of crystals [10]. Turbidometric method measures the turbidity in terms of calcium oxalate formation in synthetic urine using spectrophotometer at 620nm and crystallization inhibition measured by turbidity reduction [11]. Synthetic urine is usually prepared fresh in lab by dissolving sodium chloride 105.5 mmol/l, sodium phosphate 32.3 mmol/l, sodium citrate 3.21 mmol/l, magnesium sulfate 3.85 mmol/l, sodium sulfate 16.95 mmol/l, potassium chloride 63.7 mmol/l, calcium chloride 4.5 mmol/l, sodium oxalate 0.32 mmol/l, ammonium hydroxide 17.9 mmol/l, and ammonium chloride 0.0028 mmol/l at a constant temperature of 37˚C and pH is adjusted to 6.0 [8, 12]. Nucleation and aggregation assay involves the estimation of delay before the appearance of optically detectable crystals at 620nm [13]. Oxalates induce injury of renal epithelial cell lines is useful to perform cytotoxic effects mediated by apoptosis, cellular necrosis, release of cellular enzymes and membrane lipid peroxidation [14]. Simulation of the sedimentary crystal formation in synthetic urine provides the measurement of calcium oxalate crystal size, number and aggregation/mm^3 on hemacytometer counting chamber using polarized microscope [15]. In simultaneous flow static model (S.S.M.), two salts forming and one tested solution, taken in three separate burettes and allowed to fall slowly and drop wise into beaker in a hot water bath. Then cooled to room temperature weigh, centrifuge, dry the sediment in hot air oven and weighed the precipitate. The simultaneous flow dynamic model follows S.S.M. except continuous stirrer of reaction mixture during the flow of salt forming solutions and the tested sample. In reservoir static model (R.S.M) 50 ml of tested solution was taken in a beaker, the two salts forming solutions allowed to run into it drop wise through burettes. Thus, a reservoir of tested sample created. The rest of the operation remains same as of S.S.M. Reservoir dynamic model follows all the steps of R.S.M. except continuous stirrer of reaction mixture during the experiment [16]. The crystal growth in the silica-hydro gel is used to evaluate crystallization of urinary crystals. This technique provides systematic studies on the growth of urinary crystals. Hence, provide the information about mechanism of urinary stone formation. Simple test tubes and U-shaped tube are used for single and double diffusion gel growth technique respectively. Silica gel is used as a growth medium. Reactant solutions are mixed within the gel solution added to test tubes. After the gel formation, the supernatant / or seedling solutions are added slowly along the sides of test tubes. These solutions diffuse through the gel and interact with reactant present in the gel, leading to the growth of different types of single urinary crystals. Changes in shape, size, transparency and total mass of growing urinary crystals showed growth inhibitory / promotory effects [17]. Most of the reported *in vitro* studies describing antiurolithiatic properties of extracts are from different parts of medicinal plants (Table-1). Among the total 66 species, 35 species reported against COM, 7 against CHPD, 3 against MSUM, 9 against struvite, 7 against COM and CHPD, 1 against COM and MSUM, 1 against COM and struvite, 2 against COM, CHPD and struvite and 1 specie against COM, CHPD, MSUM and struvite (Figure-1). Indeed, the use of such extracts as complementary and alternative medicine has increased and also served as an interesting source of drug candidates [2]. However, compound targeted studies are essential to discover promising and registered natural antiurolithiatic agents for recurrent stone suppression with high efficacy and lesser side effects. Few *in vitro* phytochemical associated studies declared ascorbic acid, cerpegin, citric acid, phytic acid, tartaric acid and 8α hydroxy hedychilactone as good candidates for prophylactic management against urolithiasis (Figure-2). Ascorbic acid showed antiurolithiatic activity against COM[18]. Whereas, citric and tartaric acid (dicarboxylic acid) against CHPD crystals using crystal growth in gel technique [19, 20]. Citric acid also showed antiurolithiatic activity against COM using nucleation and aggregation assay [21]. Citric acid forms complex with calcium at pH 7.4 thereby reducing free calcium availability for COM and CHPD formation [22]. The antiurolithiatic role of citric acid is also contributed by its antioxidant action [23]. Lipid peroxidation in proximal tubule produces free radicals which injured renal tubular cell and

causes retention of COM crystals in membrane fragments to form attached stone. The stones thus formed are unable to excrete out during urination and favors urolithiasis. Antioxidant effect, by protecting membrane injury prevents COM retention and hence plays an important role to avoid calculi formation [24-26]. The high percentage of anionic groups (polyanions) adsorbed to the positively charged COM surfaces, thus masking the binding sites of COM for renal epithelial cells and also frustrates the attachment of other crystals. Excess negative charge on the adsorbed crystal(s) creates charge repulsion towards negatively charged renal epithelial cells inhibit the attachment of COM crystals with these cells. In this way, citric acid plays an important role in prophylactic management of urolithiasis by reducing nucleation, crystal growth and aggregation [27-30]. Potassium promotes urinary citrate excretion by forming potassium citrate, which alkalizes the urine by increasing pH and thus potentiates calcium-citrate-phosphate complex formation. The less bioavailable calcium and phosphate don't take part in COM and CHPD crystallization. Hyperurucosuria participates in COM formation by salting out calcium oxalate from solution. Furthermore, this condition also increases mucopolysaccharide absorption (one of the organic matrix of urinary calculi, acts as a binding agent) hence increases heterogenous nucleation and crystal aggregation. The whole process potentiates COM stone formation. The urinary alkalinization increases uric acid solubility as a response of urinary citrate excretion. This soluble urate is unable to form urate crystals and prevents the salting out of calcium oxalate by urate load, commonly known as hyperuricosuric calcium oxalate nephrolithiasis(Figure-3) [22, 31-33]. Preparations containing citric acid monohydrate are used to dissolve renal calculi and for alkalinization of urine [34]. Tartaric acid forms complex with calcium and reducing bioavailability of free calcium for COM and CHPD formation [20]. Its carboxylic acid moiety masks the COM binding sites in renal epithelial cells, reducing COM crystal growth and aggregation [29]. Cerpegin, an alkaloid isolated from the roots of *Ceropegia bulbosa* Roxb. declared similar effect against COM by colorimetric and titrimetric method [35]. The 8α hydroxy hedychilactone, a terpene from *Hedychium coronarium* J. Koenig rhizome showed activity against COM using titrimetric method [36]. Phytic acid inhibits and modulates COM using microscopic *in vitro* studies [37]. It plays an important role in COM and CHPD crystallization inhibition by forming calcium-phytic acid complex and reduces calcium bioavailability. The antioxidant property of phytic acid decreases renal cell injury ultimately decreases COM retention in renal epithelial cells. So, calcium oxalate crystals pass through urine and not participate in kidney stone formation. Phytic acid solution not only makes COM crystals defected but also modulate COM into COD. COD crystals are unable to attach with renal epithelial cells and thus pass through urine. Phytic acid inhibits intracellular calcium and phosphate accumulation followed by calcium phosphate deposits. Thus, inhibits CHPD nucleation and crystal growth by blocking calcium phosphate precipitation [37].

Generally, the extensive interactions among cells and tissues and other physiological reactions cannot be completely duplicated in a nonanimal model to claim living environment, that's why *in vivo* studies are carried out [6]. The main objective is to increase the concentration of stone forming constituents such as allantoxanamide, ethylene glycol, potassium oxonate, protein, sodium glycolate and sodium oxalate which progress to super saturation, and then crystallization followed by urolithiasis [4]. *In vivo* models provided an enormous wealth of knowledge about anatomical factors associated with calculus formation, such as identifying the initial site of crystal formation and physiological role of kidneys that would simply be impossible to obtain in any other way. Crystallization cannot be observed directly by using *in vivo* models and the mechanism of crystal deposition remains largely unexplained [7, 38]. Both models are capable of providing only limited information to the experimental scientist who wishes to know more about the manner in which urinary components affect the formation of the crystals or their attachment to the renal epithelium [39]. So, not a single model is able to encompass all aspects of pathology to find out the complete solution. More interdisciplinary research between pharmacognosists, pharmacologist and clinical investigators is needed to develop new plant derived high quality natural products to treat and prevent urolithiasis.

List of Abbreviations

COM: Calcium oxalate monohydrate / whewellite
COD: Calcium oxalate dihydrate / weddellite
CHPD: Calcium hydrogen phosphate dihydrate / brushite
MSUM: Mono sodium urate monohydrate

Table 1: List of medicinal plants with reported *in vitro* antiurolithiatic activity.

Plant	Parts used (mode of preparation)	Plant part (extract) with *in vitro* model
Whewellite (Calcium oxalate monohydrate)		
Achyranthes aspera L.	Le /St/Ro (Inf / Dec) [40]	Le (Eth) Ag and Nu [41]
Aerva lanata (L.) Juss.	Fl (NDF) [42]	Fl (Aq) Ag and Nu [42]
	Ro (NDF) [43]	Ro (Aq) SSM [43]
Ajuga iva (L.) Schreb.	NDF	Ar (Aq) SCU [15]
Ammodaucus leucotrichus Coss.		Fr (Aq) SCU [15]
Annona squamosa L.	NDF	Fr (Ju) SSM; SDM; RSM; RDM [16]
Asparagus racemosus Willd.	Ro (Dec) [40]	Ro (Eth) CM and TM [44]
Atriplex halimus L.	NDF	Le (Aq) SCU [15]
Averrhoa carambola L.	Fr (NDF) [40]	Fr (Ju) SSM; SDM; RSM; RDM [16]
Beta vulgaris L.	Ro (Ju) [40]	Le and Ro (Aq) Nu and Ag [45]
Bergenia ciliata (Haw.) Sternb.	Ro (Dec) [40]	Le (EtAc) CM and TM [10]
Bergenia ligulata Engl.	Ri (Dec) [40]	Le (Aq) CGG [46]
Bryophyllum pinnatum (Lam.) Oken.	Le (Inf / Ju) [40]	Le (Eth) Ag and Nu [41]
Butea monosperma (Lam.) Taub.	St Ba /Le (Dec); Se (Pw) [40]	Se (Aq) TM [47]

Centratherum anthelminticum (L.) Kuntze.	NDF	Se (Mth) TM [12]
Ceropegia bulbosa Roxb.	Tu (Dec) [40]	Ro (Eth) CM and TM [48]
Chamaerops humilis L.	NDF	Ba (Aq) SCU [15]
Chenopodium album L.	Le (Inf) [40]	Fr (Ju); Se (Eth) SSM; SDM; RSM; RDM [49]
Citrullus lanatus (Thunb.) Matsum. & Nakai.	Se (Inf) [40]	Fr (Ju) SSM; SDM; RSM; RDM [16]
Citrus limon (L.) Osbeck.	Fr (Ju) [40]	Fr (Ju) Nu and Ag [21]
		Fr (Ju) CGG [18]
Citrus sinensis (L.) Osbeck.	Fr (NDF) [40]	Fr (Ju) SCU [21]
Cocos nucifera L.	Fr (Wtr) [40]	Fr (Wtr) CGG [18]
Convolvulus arvensis L.	NDF	Le (Aq) Nu and Ag [13]
Erica arborea L.		Ar (Aq) SCU [15]
Erica multiflora L.		Le (Aq) SCU [15]
Globularia alypum L.		Fl and Ro (Aq) SCU [15]
Hedychium coronarium J. Koenig.	Ri (Inf) [40]	Ri (Eth) TM [36]
Herniaria hirsuta L.	Wp (NDF) [50]	Wp (Aq) Nu and Ag [50]
Hyptis suaveolens (L.) Poit.	NDF	Ap (Eth) TM [51]
Kalanchoe pinnata (Lam.) Pers.	Le (Ju) [40]	Le (Aq) Nu and Ag [52]
Kigelia africana (Lam.) Benth.	Fr (PcVn) [40]	Fr (Aq) HPM [53]
Larrea tridentata (Sessé & Moc. ex DC.) Coville.	Wp (NDF) [54]	Wp (Aq) CGG [54]
Melia dubia Cav.	Le (Dec) [8]	Le (Ac) TB [8]
Macrotyolma uniflorum (Lam.) Verdc.*	Se (Inf) [40]	Se (Aq) TM [9]
Momordica charantia L.	NDF	Le (Aq and Eth) Nu and Ag [55]
Mimosa pudica L.	Le (Ju); Ro (Dec) [40]	Wp (Aq) CGG [18]
Mucuna pruriens (L.) DC.	Wp (NDF) [56]	Wp (Eth) TB [56]
Musa × *sapientum* L.	NDF	St (Aq) CGG [18]
Nigella sativa L.	Fr / Se (Inf) [40]	Se (Mth) TM [47]
Ocimum gratissimum L.	Wp (Dec) [40]	Le (Eth) SCU and TB [57]
Persea americana Mill.	Le (Dec) [40]	Fr (Ju) SSM; SDM; RSM; RDM [16]
Phyllanthus niruri L.	Le (Dec) [40]	Le (Aq) SCU [58]; TB [11]
Semecarpus anacardium L.f.	Wp (NDF) [59]	Se (Chl) TM [59]
Sida acuta Burm.f.	Le (NDF) [60]	Le (Eth) TB [60]
Stipa tenacissima L.	NDF	Le (Aq) SCU [15]
Terminalia chebula Retz.	Ba (Inf) [40]	Fr (Chl)TM [59]
Tetraclinis articulata (Vahl) Mast.	NDF	Le (Aq) SCU [15]
Tinospora cordifolia (Willd.) Miers.	Le / St (Ju) [40]	St (Chl)TB [59]
Rotula aquatica Lour.	Ro / St (Dec) [40]	Ro (Aq) Nu and Ag [61]
Tribulus terrestris L.	Fr/ Le / Se / Ro (Dec /Inf) [40]	Fr (Aq) CGG [46]; ORC [14]
Zea mays L.	Zmh (Dec/ Inf [40]	Zmh (Aq) HPM [62]
Brushite (Calcium hydrogen phosphate dihydrate)		
Acacia raddiana Savi.	NDF	Ba (Aq and Eth) SCU [63]
Achyranthes aspera L.	Le /St/Ro (Inf / Dec) [40]	Ro (Aq) CGG [64]
Aerva lanata (L.) Juss.	Ro (NDF) [43]	Ro (Aq) CGG [43] SSM [43]
	Sh (NDF) [65]	Sh (Aq) CGG [65]
Averrhoa carambola L.	Fr (NDF) [40]	Fr (Ju) SSM; SDM; RSM; RDM [16]
Ananas comosus (L.) Merr.	Fr (Ju) [40]	Fr (Ju) CGG [18]
Borassus flabellifer L.	NDF	Fr (Ju) CGG [18]
Chenopodium album L.	Le (Inf) [40]	Fr (Ju); Se (Eth) SSM; SDM; RSM; RDM [49]
Citrus limon (L.) Osbeck.	Fr (Ju) [40]	Fr (Ju) CGG [18]
Citrullus colocynthis (L.) Schrad.	Wp (NDF) [40]	Fr (Aq) SCU [63]
Citrullus lanatus (Thunb.) Matsum. & Nakai.	Se (Inf) [40]	Fr (Ju) SSM; SDM; RSM; RDM [16]
Cocos nucifera L.	Fr (Wtr) [40]	Fr (Wtr) CGG [18]
Ensete superbum (Roxb.) Cheesman.	Ro (Ju); Se (Pw) [40]	Aq (dec) CGG [66]
Mimosa pudica L.	Le (Ju); Ro (Dec) [40]	Wp (Aq) CGG [18]
Persea americana Mill.	Le (Dec) [40]	Fr (Ju) SSM; SDM; RSM; RDM [16]
Tamarindus indica L.	Fr / Le (Dec) [40]	Fr (Aq) CGG [20]
Vitis vinifera L.	Fr (Ju) [40]	Fr (Aq) CGG [18]
Zea mays L.	Zmh (Dec/ Inf [40]	Zmh(Aq)HPM [62]
MSUM (Mono sodium urate mono hydrate)		
Aerva lanata (L.) Juss.	Ro (NDF) [43]	Ro (Aq) CGG [67]
Boerhaavia diffusa L.*	Ro (Dec) [40]	
Boswellia serrata Roxb. ex Colebr.	NDF	Gum resin (Aq) CGG [20]
Ceiba pentandra (L.) Gaertn.		Ba (Aq) CGG [68]
Rotula aquatica Lour.	Ro / St (Dec) [40]	Ro (Aq) CGG [67]
Struvite (Ammonium magnesium phosphate hexahydrate)		
Acacia raddiana Savi.	NDF	Ba (Aq) SCU [63]
Aerva lanata (L.) Juss.	Ro (NDF) [43]	Ro (Aq) CGG [69]
Boerhaavia diffusa L.*	NDF	Ro (Aq) CGG [70]
Citrullus colocynthis (L.) Schrad.	Wp (NDF) [40]	Ba (Eth) SCU [71]

		Fr (Aq) SCU [63]
Citrus limon (L.) Osbeck.	Fr (Ju) [40]	Fr (Ju) CGG [18]
Citrus medica L.	NDF	Fr (Ju) CGG [72]
Cocos nucifera L.	Fr (Wtr) [40]	Fr (Wtr) CGG [18]
Commiphora wightii (Arn.) Bhandari.	NDF	Fr (Aq) CGG [73]
Mentha spicata L.	Le (Dec) [40]	Le (Aq) CGG [18]
Musa × *sapientum* L.	NDF	St (Aq) CGG [18]
Pistacia lentiscus L.	Wp (Dec) [40]	Ba (Eth) SCU [71]
Raphanus sativus L.	Ba / Le (Ju) / Ro (Inf) /Se (Pw) [40]	Ro (Aq) CGG [18]
Rhus tripartita (Ucria) Grande.	NDF	Ba (Eth) SCU [71]

Keys: Ac: acetone ;Ag and Nu= Aggregation and nucleation; Aq: aqueous ; Ar: aerial part; Ba: bark; CGG= crystal growth in gel; CM= colorimetric method; Chl: chloroform; Et Ac: ethyl acetate; Eth; ethanolic; Fr: fruit; HPM: homogenous precipitation method; Le: leaves; Mth: methanol; NDF: no data found; Nu & Ag: nucleation and aggregation assay; ORC= oxalate induced renal tubular epithelial cell injury; Pw: powder; Ro: root; SCU= Simulation of the sedimentary crystal formation in synthetic urine; Se: seeds; Sh: shoot; TB= turbidometric method; TM= Titrimetric method; Zmh: Zea mays hair; *= plants not found in the electronic database *The Plant List* - a working list of all plant species created by Royal Botanical Gardens, Kew and Missouri Botanical Garden.

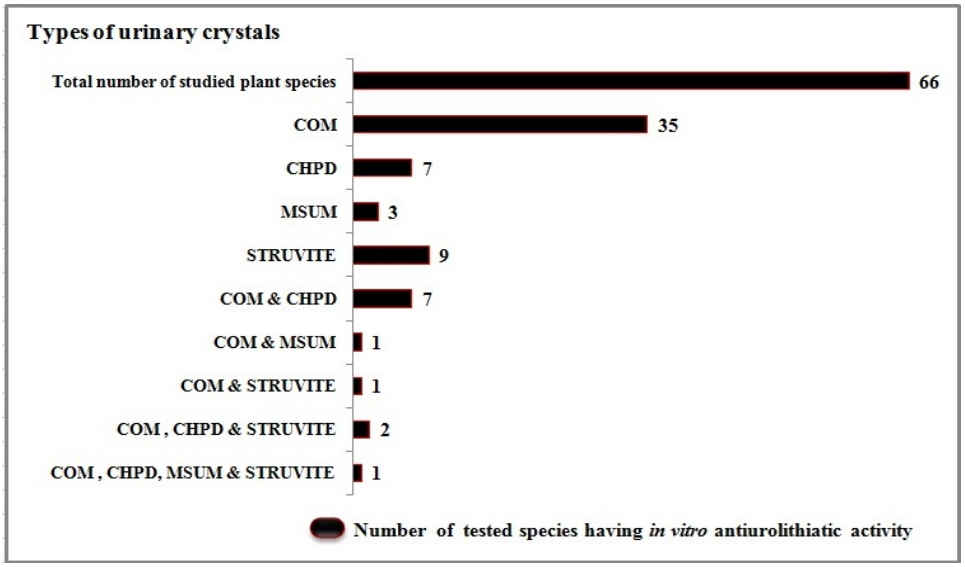
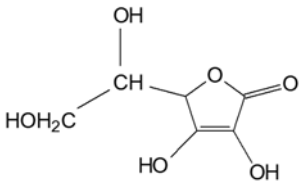
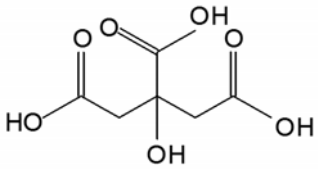


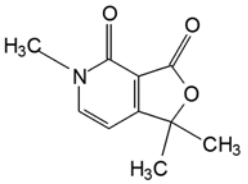
Fig 1: Number of studied plant species having *in vitro* antiurolithiatic activity against different type of urinary crystals. COM: calcium oxalate monohydrate; CHPD: calcium hydrogen phosphate dihydrate; MSUM: monosodium urate monohydrate



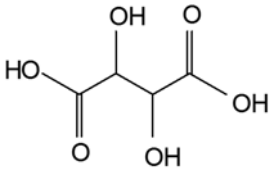
Ascorbic acid ($C_6H_8O_6$)



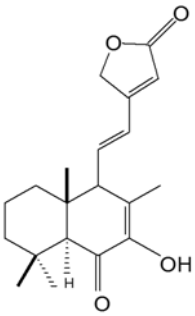
Citric acid ($C_6H_8O_7$)



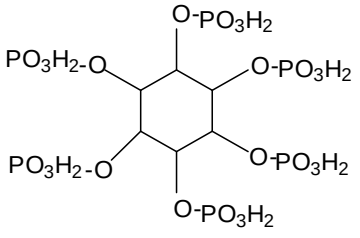
Cerpegin ($C_9H_9NO_3$)



Tartaric acid ($C_4H_6O_6$)



8α hydroxy hedychilactone



Phytic acid ($C_6H_{18}O_{24}P_6$)

Fig 2: Reported phytochemical compounds having *in vitro* antiurolithiatic activity.

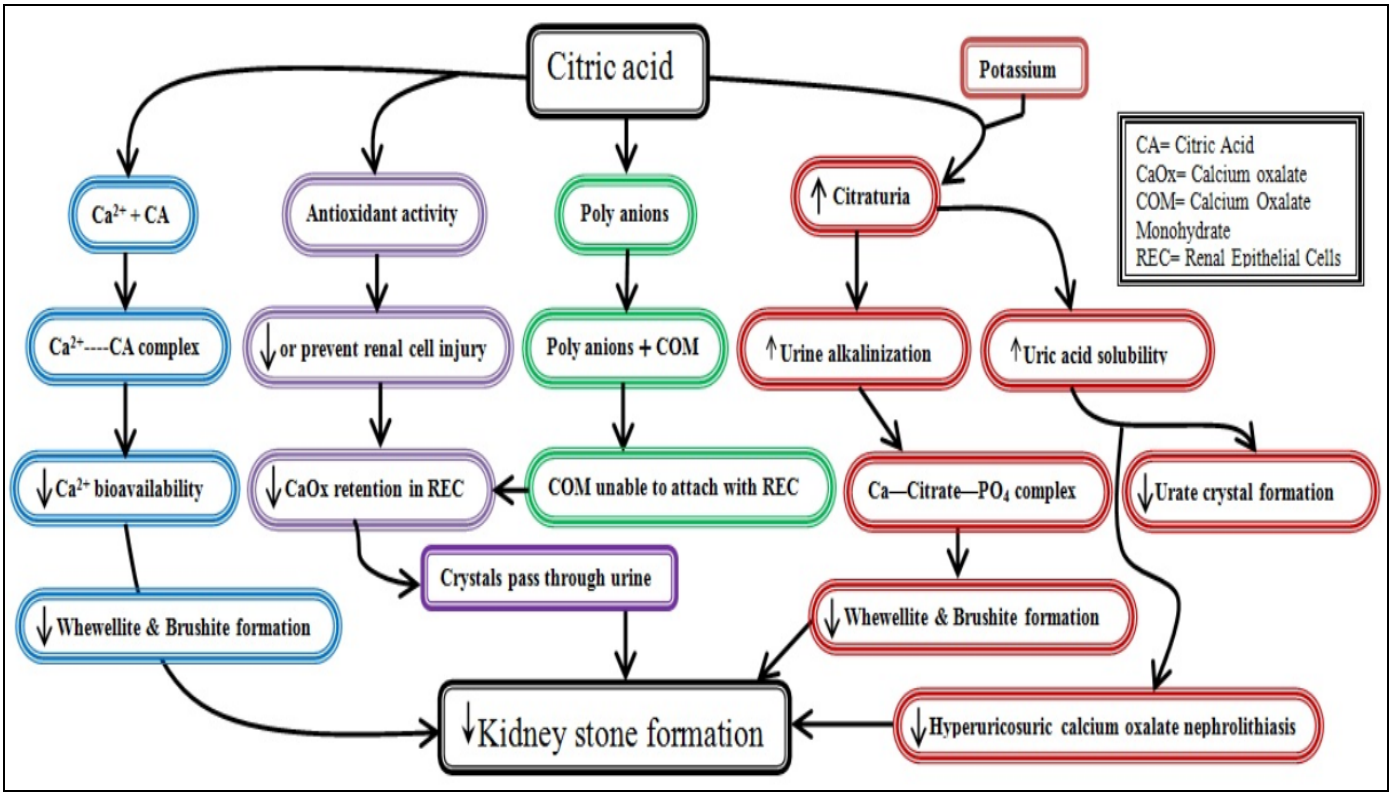


Fig 3: Prophylactic antiurolithiatic mechanism of citric acid.

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