DISCUSSION
Urolithiasis can be traced to the earliest antiquity of human history. Urinary tract stones are a worldwide problem, sparing no geographical, cultural or racial groups (Moe, 2006). Renal stone disease has a worldwide prevalence between 2 and 20% (Johri et al., 2010) with a peak incidence between 20 and 40 years of age (Miyoka and Monga, 2009). The disease is also associated with high cost to the society because of the high prevalence of the disease and high recurrence rates (Johri et al., 2010; Lotan, 2009). Renal stone disease is a multifactorial disorder based on dietary, environmental and genetic factors (Johri et al., 2010) with a prevalence rate of 15% in India (Rizvi et al., 2002). Dehydration is one of the risk factors linked to kidney-stone disease, and studies suggest global warming will exacerbate this effect. The researchers predict that by 2050, higher temperatures will cause an additional 1.6 million to 2.2 million kidney-stone cases, representing up to a 30 percent growth in some areas (Brikowski et al., 2008). Recently, estimates from computer models predicted up to a 10% increase in the prevalence rate in the next half century secondary to the effects of global warming, with a coinciding 25% increase in health-care expenditures (Fakheri and Goldfarb, 2011).

Kidney stone formation is a complex process and the result of a cascade of events, including crystal nucleation, growth, and aggregation, and crystal retention within the renal tubules (Khan, 1997). Crystalluria is common while stone formation is not. Only pathological changes in the kidneys including renal injury and dysfunction can accomplish crystal retention (Khan, 2006). Calcareous stones are still by far the most common uroliths, accounting for more than 80% of stones. Uric acid stones represent about 5-10%, trailed by cystine, struvite and ammonium acid urate stones (Moe, 2006).
Discussion

There is much interest among physicians and patients to identify effective measures to promote stone passage, stone dissolution and stone prevention. Management of stone disease depends on the size and location of the stones. Stones less than 5 mm in diameter have high chance of passage; those of 5 - 7 mm have a modest chance (50%) of passage; and those greater than 7 mm almost always require surgical intervention (Coe et al., 2005). Currently this serious problem can be treated with extracorporeal procedures such as extracorporeal shock wave lithotripsy (ESWL), endourological procedures such as ureterorenoscopy (URS) or percutaneous nephrolithotomy (PCNL) and the combination of these techniques (Gurocak and Kupeli, 2006). However, compelling data showed that exposure to ESWL may cause acute renal injury, a decrease in renal function and increase in stone recurrence. Furthermore, traumatizing effects of shock waves, persistent residual stone fragments after ESWL and a possibility of infection pose serious problems to be taken into consideration (Atmani, 2003). ESWL is also reported to be associated with long term medical effects as diabetes mellitus and hypertension. Apart from surgical interventions, dietary and some therapeutic interventions are also recommended like thiazides, potassium citrate, allopurinol (Tiselius, 2003; Sayer et al., 2010; Heilberg and Schor, 2006; Moe, 2006; Atmani, 2003). The role of probiotics, exploring the potential of the bacteria Oxalobacter formigenes is also being examined for treatment of urolithiasis (Tracy and Pearle, 2009).

The treatment and prevention of kidney stones has considerably revolutionised during the last two decades by combination of dietary procedures, surgical treatments and medicaments, but as mentioned above, side effects of these methods and persistence of recurrence remain as problems to overcome. Thus, an adjunct to these conventional
methods as phytotherapy is highly recommended. Medicines plants have been known for millennia and highly esteemed all over the world as a rich source of therapeutic agents for the prevention of diseases and ailments.

The marketed composite herbal formulations, Cystone (Himalaya Drug Company, India), Neeri (Aimil Pharmaceuticals, India), Uriflow (Bioneutrix Labs), Urite (Aimil Pharmaceuticals, India), Culdisol (Ganga Pharmaceuticals, India), Calcury (Charak Pharmaceuticals, India), Chandraprabhati (Baidyanath, India) and Culin Forte (Alopa Herbal), have been used worldwide to dissolve urinary calculi in kidney and urinary bladder. These formulations are mixture of various plant extracts in some form or the other. A large number of plant species have been described in many pharmacopoeias all over world as remedies for urolithiasis. Till date, various plant extracts have been studied to reduce the incidence of calcium stone deposition both in vitro and in vivo (Butterweck and Khan, 2009; Atmani, 2003) but the identification of naturally occurring calcium oxalate (CaOx) inhibitory biomolecules from plants was hampered in past by limitations in identification method. Thus, the present study is aimed at investigating the inhibitory potency of Tribulus terrestris on calcium oxalate crystal nucleation and growth both in vitro and in vivo along with the isolation and characterization of new biologically active compounds from Tribulus terrestris.

5.1 Antiurolithiatic potency of aqueous extract of Tribulus terrestris

_in vitro and reduction of oxalate induced renal epithelial cell injury_

Tribulus terrestris, commonly called as “gokhru” is commonly used in ayurveda for its medicinal value. The plant is an active constituent of various marketed antiurolithiatic herbal formulations like Cystone, Neeri and Uriflow. In the present
study, the inhibitory potency of *T. terrestris* was evaluated *in vitro* on calcium oxalate and calcium phosphate crystallisation. The supersaturation of urine with CaOx, the most common component of kidney stones, is an important factor in crystallization, with later factors being nucleation, growth and aggregation. Thus, if supersaturation or later steps in crystallization can be prevented, then lithiasis can be avoided (Beghala *et al.*, 2008). With respect to calcium oxalate crystallization, the aqueous extract of the plant was quite effective in inhibiting both nucleation and growth of CaOx crystals effectively to the tune of ~100% with 1000µg/ml plant sample. Our studies are in accordance with the studies already done to establish the antiurolithiatic potency of *Tribulus terrestris* on the growth CaOx crystals using double diffusion gel growth technique (Joshi *et al.*, 2005b). Antiurolithiatic activity of *Origanum vulgare*, tested on nucleation and growth of CaOx crystals by Khan *et al.*, was found to be in a concentration dependent manner as measured by turbidity in nucleation phase and aggregation phase (Khan *et al.*, 2011).

Crystals can be retained at many sites in the kidneys through the size enhancing process of aggregation and by attachment to the renal epithelium. It has been suggested that in idiopathic stone formers, calcium phosphate (CaP) deposits originate in the basement membrane of the Loops of Henle and from there continuously grow outward reaching the papillary surface (Evan *et al.*, 2003). The CaP deposits on papillary surface then become focal points for the development of CaOx kidney stones (Khan, 2006). The extract was able to demineralise calcium and phosphate ions effectively though it was not as effective in initial mineralisation and growth of preformed mineral phase of calcium phosphate. A study was performed to evaluate
the crystal dissolution potency of extracts of *Rotula aquatica*, *Commiphora wightii* and *Boerhaavia diffusa* against basic calcium phosphate, calcium pyrophosphate and monosodium urate monohydrate (Raut *et al.*, 2008). Experimental studies carried out on *Crateva nvala*, *Tribulus terrestris* and *Dolichos biflorus* showed them to be effective in dissolving phosphate type of calculi in an *in vitro* model (Pramod *et al.*, 1981). In our lab too, aqueous extracts of various plants have been tested for their antiurolithiathic potency against CaP and CaOx *in vitro* crystallization as *Dolichos biflorus*, *Trachyspermum ammi* (Dijama *et al.*, 2006), *Achyranthes aspera* (Aggarwal *et al.*, 2010), *Terminalia arjuna* (Chaudhary *et al.*, 2010) and *Tamarindus indica* (Chaudhary *et al.*, 2008) and these plants were found to be potent candidates against urolithiasis.

While the physical chemistry of stone formation has been intensively studied during the last decade, it has become clear that the pathophysiology of the renal stone disease cannot be explained by crystallization processes only. In recent years, evidence has emerged that the cells lining the renal tubules can have an active role in creating the conditions under which stones may develop. Since, these mechanisms are difficult to study *in vivo*, cultured renal tubular epithelial cells are a good option for the study of physiological and cell biological processes that are possibly linked to stone disease (Verkoeten *et al.*, 1991). Evidence that the association of crystals with renal tubule cells is involved in urolithiasis came from the scanning and transmission electron microscopy images which showed the attachment of the crystals to the luminal surface and also inside the cells (Leiske *et al.*, 1992). It has been suggested that oxalate not only promotes stone disease by providing an appropriate environment for crystal formation but the ion itself may affect the renal epithelium to predispose the
tissue crystal retention leading to the release of renal enzymes (Verkoelen et al., 1997; Thamilselvan et al., 2003). This explains the significant release of LDH and decrease in cell viability in the cells exposed to 1mM oxalate ions which correlates with other studies done indicating cellular damage on exposure to oxalate or CaOx crystals (Koul et al., 1996; Scheid et al., 2000). High level of oxalate cause a variety of changes in the renal epithelial cells, such as an increase in free radical production and a decrease in antioxidant status, followed by cell injury and cell death. These changes are significant predisposing factors for the facilitation of crystal adherence and retention (Khan, 1995; Moriyama et al., 2007). In the study with NRK-52E, Tribulus terrestris proved to have a protective effect towards the renal epithelial cells again in a concentration dependent manner. When NRK-52E cells were injured by exposure to oxalate for 72 h, the plant extract prevented the injury in a dose-dependent manner. The mechanism of inhibition/reduction in the injury needs to be studied further. Studies have shown that inhibition of the inflammatory response induced by injury due to crystal formation helps in restoring normalcy. Recently plants including Herniaria hirsuta and Phyllanthus niruri are being explored for their antiurolithiatic property on the basis of their usage in the traditional medicine. Herniaria hirsuta, a plant from Morocco is also known to exhibit the antilithiatic activity. The adhesion of the radioactive CaOx crystals to the Madin Darby canine kidney (MDCK) cells was studied in the presence and the absence of the aqueous extract. CaOx crystal binding to the cells was inhibited by the extract in a concentration dependent manner (Atmani et al., 2004). In vitro effect of an aqueous extract of Phyllanthus niruri L., a plant used in Brazilian folk medicine for the treatment of urolithiasis, exhibited a potent and effective non-concentration-dependent inhibitory effect on the CaOx crystal
internalization on a model of CaOx crystal endocytosis by Madin-Darby canine kidney cells. This response was present even at very high (pathologic) CaOx concentrations and no *Phyllanthus niruri* L.-induced toxic effect could be detected (Campos and Schor, 1999). Beghalia *et al.*, 2008 have suggested in studies using certain Algerian medicinal plants that the herb extract may contain substances that inhibit the growth of CaOx crystals. This property of plant extracts could be important in preventing kidney stone formation; the agglomeration of particles is a critical step in urinary stone formation, as larger crystals are less likely to pass spontaneously in urinary tract (Kok and Khan, 1994). Beghalia *et al.*, 2008 postulated that the plant extracts may contain substances that inhibit CaOx crystal aggregation. Binding of the crystal to the renal epithelial surface is blocked by active biomolecules of the plants (Atmani *et al.*, 2004). This could explain a decrease in the LDH release seen in the cells treated with the plant extract as compared to those treated with oxalate alone.

5.2 *In vivo* antiurolithiatic properties of aqueous extract of *Tribulus terrestris*

In the view of its medicinal use, *Tribulus terrestris* fruit extract was studied to evaluate its antiurolithiatic potential using different models. As the aqueous extract was found to be effective in inhibiting crystallization *in vitro* and proved to be protective towards oxalate induced renal tubular epithelial cell injury, hyperoxaluric rat model was used to study the effect of aqueous extract of *Tribulus terrestris* on crystal deposition and its consequences *in vivo* in prophylactic and curative regimen. Cruzan *et al.*, 2004 reported significant strain differences exist with respect to sensitivity to ethylene glycol and its metabolites, including oxalate. Curiously, rats
that are apparently more sensitive to ethylene glycol (i.e., the Wistar strain) have calcium oxalate crystal deposition in renal tissues within 5 to 7 days of ethylene glycol treatment (Huang et al., 2000; 2003), while the same experimental regimen does not produce crystals in Sprague-Dawley rats until 10 to 14 days of treatment (Khan et al., 1989; Thamilselvan et al., 1997). Whether the Sprague-Dawley strain of rats is less sensitive to the toxic effects of oxalate than the Wistar strain remains speculative, but the several lines of experimental evidence discussed above suggests it is an intriguing possibility.

Urinary supersaturation with respect to stone forming constituents is generally considered to be one of the causative factors in calculogenesis and the biochemical mechanism for this process is related to an increase in the urinary concentration of oxalate (Gilhotra and Christina, 2011). Hyperoxaluria is being induced using ethylene glycol alone or in combination with ammonium chloride (Lee et al., 2011). Ethylene glycol disturbs oxalate metabolism by way of increasing the substrate availability thus leading to hyperoxaluria, while ammonium chloride accelerates the process through urinary acidification (Khan, 1997; Atmani et al., 2003). In a study performed by Yamaguchi et al., the combination of high doses of EG (0.4%, 0.8%) and NH₄Cl (1%) in the drinking water induced crystalluria and hyperoxaluria, along with calcium oxalate deposits in the kidney. In addition, deterioration of renal function was observed, especially after 11 days (Yamaguchi et al., 2005). In the present study, 0.4% EG was supplemented with 1% NH₄Cl for 15 days of prophylactic regimen and in curative regimen, for last 13 days, rats were treated with 0.4% EG only leading to a 28 days of treatment period. Male rats were selected to induce urolithiasis because the urinary system of male rats resembles that of humans (Vermeulen, 1962) and earlier
studies have shown that the amount of stone deposition in female rats is significantly less (Prasad et al., 1993). Intraperitoneal administration of the extract was used as it may lead to adequate absorption of active phytoconstituents of the plant (Harlalka et al., 2007; Bashir et al., 2010).

Lithogenic treatment caused decrease in body weights similar to as found in other studies (Touhami et al., 2007; Bashir et al., 2010) and impairment of renal functions of untreated rats as evident from the markers of glomerular and tubular damage: raised serum urca and serum creatinine, reduced creatinine clearance and increased urinary enzyme loss. These effects were dose dependently prevented in the animals receiving a simultaneous dosage of aqueous extract of *Tribulus terrestris* in prophylactic regimen and cured in the animals receiving a concurrent treatment of the extract (50 mg/kg body wt and 100 mg/kg body wt).

Specific proteins excreted in the urine after injury to particular segments of the nephron can serve as biomarkers for assessing the site and severity of renal damage. Previously used biomarkers can be broadly classified into the following three categories: 1) enzymes: alanine aminopeptidase, alkaline phosphatase, \( \gamma \)-glutamyl transpeptidase, N-acetyl-\( \beta \)-D-glucosaminidase, cathepsin B, lysozyme, and lactate dehydrogenase; 2) low-molecular-weight proteins: \( \beta_2 \)-microglobulin, \( \alpha_1 \)-microglobulins, and retinol-binding protein; and 3) kidney-derived antigens: glutathione-S-transferase (GST), clusterin, CYR-61, Neutrophil gelatinase-associated lipocalin (NGAL), and F-actin (Vaidya et al., 2006). For several years, studies have demonstrated that excreted urinary enzymes may be useful biomarkers for evaluation and diagnosis of tubular dysfunction or injury. These markers suggests that tubular damage most likely precedes glomerular damage and therefore reinforcing
observations that urinary enzyme excretion can be used as early markers (Gatua et al., 2011). Alkaline phosphatase (ALP) and lactate dehydrogenase (LDH) are two cytosolic enzymes and their higher activity in the extracellular fluid indicates cell lysis. It has been shown that both oxalate and calcium oxalate crystals independently increase free radical generation in a time and concentration dependent manner (Byer and Khan, 2005; Greene et al., 2005; Scheid et al., 1996) and that oxalate alone increases oxidative cell injury while calcium oxalate crystals potentiate injury (Thamilselvan et al., 2000). Oxalate-induced oxidative stress disrupts structural integrity of the renal epithelial membrane (Thamilselvan et al., 2009). The rats induced with ethylene glycol and ammonium chloride showed a significant elevation of renal injury marker enzymes (ALP and LDH) in both prophylactic and curative regimen, though the increase in curative model was more than in prophylactic model. This is because EG results in many other toxic metabolites in addition to oxalic acid and exposure to these toxins for longer duration would result in higher order of renal injury (Khan et al., 2006). The enhanced urinary excretion of injury marker enzymes in urolithic rats suggests damage to the brush border membrane of renal tubules, which appears to associate with the retention and deposition of crystals in the kidney (Khan et al., 1992). Administration of different doses of the aqueous extract of Tribulus terrestris in prophylactic and curative regimen (50 and 100 mg/kg body wt) had profound effect on reducing the urinary enzyme excretion, in the form of decreasing ALP and LDH activity thus preventing the nidus formation for nucleation and thereby minimizing the extent of tubular dysfunction (Vidya and Varalakshmi, 2000; Soundararajan et al., 2006). This shows that the extract which was able to reduce renal injury in vitro, potentiates to protect and heal the renal damage in vivo.
too, which further provides sites for CaOx deposition (Khan et al., 1992). Thus, the probable mechanism by which *Tribulus terrestris* have reduced renal injury might be due to its ability to inhibit CaOx crystals causing this damage.

The kidney function is affected in urolithiasis, since lowering of the glomerular filtration rate (GFR) is observed due to the obstruction to the outflow of the urine by calculi deposited along the urinary system. Thereby, the waste products particularly nitrogenous substances such as urea, creatinine and uric acid, accumulate in blood (Ghodkar, 1994). Also increased lipid peroxidation and decreased levels of antioxidant potential have been reported in the kidneys of rats supplemented with a calculi producing diet (Sumathi et al., 1993; Saravan et al., 1995). Oxalate, being the precursor molecule to induce lipid peroxidation, further causes renal tissue damage by reacting with polyunsaturated fatty acids in the cell membrane (Divakar et al., 2010). In this scenario, marked renal damage is observed in calculi-induced rats ascribed by virtue of the elevated serum levels of creatinine and urea in prophylactic and curative regimen. However, normalization as caused in the levels of both serum urea and creatinine by the fruit extract of *T. terrestris* leads to accelerate the process of dissolving preformed crystals and interrupts the process of crystal aggregation and deposition along the urinary system, as the plant extract has proved to be better in reverting the damage caused by calculi induction. The significant lowering of serum levels of the accumulated waste products may be attributed to the enhanced GFR, antioxidant property (Kamboj et al., 2011) and diuretic potential of *Tribulus terrestris* (Al-Ali et al., 2003; Wright et al., 2007).

Clinically, creatinine clearance (CrCl) is a useful measure for determining renal functioning (Worcester et al., 2006). Renal dysfunction diminishes the ability to filter
creatinine and increases serum creatinine levels, thus decreasing CrCl. The impairment of renal functioning after exposure to EG and NH₄Cl is an outcome of CaOx crystals deposition in renal tissue. Administration of the plant extract in different doses was able to restore the renal functioning by preventing the elevation of serum levels of creatinine (Prophylactic regimen) and also reverting the damage caused on lithogenic treatment to the kidney (Curative regimen). It has been found that the external prophylactic agents restore renal functioning by maintaining creatinine clearance and serum urca levels in hyperoxaluric rats (Dijarnia et al., 2008). Microscopic examination of kidney sections derived from calculi induced urolithic rats showed polymorphic irregular crystals deposits in the tubules accompanied by cast formation which causes dilation of proximal tubules which might be attributed to oxalate formation (Soundarajan et al., 2006) on exposure of 15 days. Recent in vitro studies have suggested that proximal tubule cells, when compared to distal tubule or collecting duct cells, are more sensitive to the toxic effects of both oxalate and calcium oxalate at pathological level (Thamilselvan et al., 1999). Studies have shown that crystal formation results in cell damage and cell detachment from the basement membrane and the released degradation products like CD44, which further promote nucleation of crystals (Hackett et al., 1990; Verkoelen et al., 1998; Verkoelen, 2006). A severe effect was observed in rats which were exposed to lithogenic treatment for 28 days. Along with the damage to the proximal tubules, glomerular alterations like glomerular congestions and peritubular congestions were also observed. Our results were in corroboration with the studies done in rats and humans with calcium oxalate stone disease (Evan et al., 2010; Worcester et al., 2006). Glomerular damage leads to improper filtration of biomolecules from the blood into urine, thus leading to
proteinuria (Worcester et al., 2006), whose normal cut off in physiological condition is 60kDa (Gupta, 2009). Direct injury or stimuli from various other forms of renal dysfunction like EG toxicity activate tubular cells which, in turn, interact with interstitial tissue elements and inflammatory cells, causing further pathologic changes in the renal parenchyma leading to a feed-forward loop of kidney injury (Leth and Gregerson, 2005; Hodgkins and Schnaper, 2011). Renal interstitial cells play an important role in renal function and renal diseases. The interstitial cells, besides taking part in the modelling of the extracellular matrix, play a role in the production of regulatory substances and in immune responses and also in the production of erythropoietin (Kaisling et al., 1996). Gentamicin induced nephrotoxicity also leads to calculi induction with similar histological changes as observed in our study under ethylene glycol and ammonium chloride exposure (Harlalka et al., 2007; Raju et al., 2011). Administration of Tribulus terrestris to EG and NH₄Cl exposed rats, prevents supersaturation of CaOx and also decreased their deposition in renal tubules. By decreasing the vascular congestion of glomerulus, the plant was able to restore back the normal functioning of the nephrons thus reducing the proteinuria. The plant was effective in reducing the trapping of the crystals in the tubules in both prophylactic and curative regimen. As the interstitial cell population increased on administration of Tribulus terrestris, the probable mechanism of action can be through crystal removing ability of macrophages through phagocytosis (Okada et al., 2010), with the plant having the role in increasing the accumulation of macrophages.

The analysis of crystalluria after 15 days of treatment with CaOx stone inducing agents showed that untreated animals excreted abundant and larger crystals than treated animals. Crystalluria could occur in both healthy and stone forming subjects
though agglomeration of particles is considered a crucial step in urinary stone formation because larger crystal aggregates are less likely to pass spontaneously from the urinary tract (Kok and Khan, 1994) and stone forming individuals tend to excrete larger and aggregated particles than the former (Robertson et al., 1969). In the present study, upon exposure to lithogenic treatment (EG + NH₄Cl) for 15 days and 28 days, calcium oxalate and phosphate crystals were observed. Morphological studies have provided evidence that idiopathic calcium oxalate (CaOx) stones develop on subepithelial plaques of calcium phosphate (Escobar et al., 2008), thus can serve as a nidus for CaOx crystals growth. Crystal aggregation is promoted by viscous binding, implying that crystal-foreign compounds with multiple binding sites attach to crystal surfaces and act as a kind of glue (Tsujihata, 2008). Tribulus terrestris extract dose reduced supersaturation of CaOx crystals in the urine as compared to urolithic rats significantly. In prophylactic regimen, the extract (100 mg/kg body wt) was able to break the crystals induced by lithogenic treatment into many small crystals, thus facilitating easy expulsion from the kidneys as this is reported to be a mechanism for preventing stone formation (Pareta et al., 2011). Larger crystals have a greater chance of being trapped within renal tubules, whereas smaller crystals can be flushed easily from the kidneys (Atmani et al., 2003), hence, this effect could be advantageous in preventing urinary stone formation, as agglomeration is a crucial step in kidney stone formation. In curative regimen (treatment period of 28 days), administration of the plant was quite effective as a significant reduction in the number of crystals was also observed in the urine of these rats. In consistence with studies done with Bergenia lingulata, an antiurolithiatic plant of South Asia (Bashir and Gilani, 2009), decrease in crystal count serve a two way benefit. Firstly, it decreases supersaturation, a
prerequisite for stone formation and secondly, it reduces the site of crystal growth and aggregation, proving to be beneficial in treating the damages caused by lithogenic treatment.

From the above results, it can be emphasized that aqueous extract of Tribulus terrestris has an ability to prevent and reduce the crystal deposition in kidneys. The extract is effective in reducing the renal tissue injury, decreasing the crystal size thus facilitating easy expulsion and restoring normal kidney architecture.

5.3 Proteins from the fruits of *Tribulus terrestris*

Various CaOx growth inhibitors mostly proteins like nephrocalcin, uropontin and citrate and glycosaminoglycans (GAGs) have been reported in humans to play an important role in renal stone diseases for several decades (Coe *et al.*, 1991; Leiske *et al.*, 1999). Many plants are also known to produce CaOx as crystalline deposits, having an organic matrix constituting of different proteins (Nakata, 2003; Li *et al.*, 2003). Recently, it was observed that water soluble protein matrix associated with calcium oxalate crystals from bean seed coat (*Phaseolus vulgaris*) contains many polypeptides out of which two proteins were isolated and they showed strong inhibition towards nucleation of CaOx in a concentration dependent manner. It was also shown that the isolated proteins modified the morphology of CaOx crystals mainly at \{1 2 0\} face (fastest growing face) (Jáuregui-Zúñiga *et al.*, 2005). A well known CaOx inhibitor, citrate, has also shown to slow the growth at \{1 2 0\} face (Qui *et al.*, 2004). Calsequestrinlike calcium binding protein was isolated from calcium accumulating cells of *Pistia stratiotes* (Franceschi *et al.*, 1993). Recent studies done in our lab has also reported two anticalcifying proteins each from *Dolichos biflorus* (Bijarnia *et al.*, 2009) and *Trachyspermum ammi* (Kaur *et al.*, 2009) with high content
of acidic amino acids. The purified protein from *Trachyspermum ammi* was also found to be effective against induced kidney stones *in vivo* (Kaur *et al.*, 2009). Aspartic acid and glutamic acid are quoted to play a vital role in the antilithiatic potency of *Tribulus terrestris* by disintegrating the stone outer shell, according to the datasheet of an herbal composition “Uriflow” marketed by Bioneutrix. So, a CaOx inhibitory protein from the fruits of *Tribulus terrestris* with strong anticalcifying properties *in vitro* was isolated, purified and characterized.

In the present study, an antilithiatic protein was isolated from the fruits of *Tribulus terrestris* (TTP) inhibiting calcium oxalate crystallisation. The protein was purified through ammonium sulphate precipitation, anion exchange and finally molecular sieve chromatography. This isolated purified protein had a molecular weight of ~60kDa. The diuretic potency of *Tribulus terrestris* has been explored by various groups *in vitro* and *in vivo* but the purification of active biomolecules is still not done (Sangeeta *et al.*, 1994; Anand *et al.*, 1994). The inhibitory potency of the purified protein was found to be as high as 78.3% and amino acid analysis of the most potent fraction revealed the protein to be tyrosine rich. The purified in-gel tryptic digested protein when subjected to MALDI-TOF for peptide mass fingerprinting analysis and MASCOT search engine showed the maximum similarity (17% sequence coverage) with carotenoid cleavage dioxygenase 7 (CCD7) of *Arabidopsis thaliana* with a molecular weight of 65kDa and pI 6.01. BLASTp analysis of CCD7 showed similar hits, further verifying the hit obtained from MASCOT. The presence of an EF hand domain in a homologous protein indicates that this protein probably imparts its inhibitory effect by binding to calcium ions and thus minimizing the availability of calcium for the formation of CaOx crystals.
CCD7 belong to a family of dioxynones which possess characteristic five conserved histidines spread throughout their primary protein sequence, secondly, they require Fe$^{2+}$ ions thought to be coordinated by the five histidine residues and they contain a conserved polypeptide segment at their carboxy terminus that minimally constitutes a signature sequence for the family (Redmond et al., 2001). The oxidative cleavage of carotenoids leads to the production of apocarotenoids and is catalyzed by a family of carotenoid cleavage dioxygenases (CCDs). CCDs often exhibit substrate promiscuity, which probably contributes to the diversity of apocarotenoids found in nature (Auldridge et al., 2006). CCD7 cleaves its substrates specifically at the 9, 10 double bond asymmetrically (Schwartz et al., 2004). With β-carotene as a substrate, which is also called as provitamin A, CCD7 produces one β-ionone product playing the role of pollinator attractant, fruit or vegetable flavor and the C27 product, 10'-apo-β-carotenal that is required for the normal inhibition of shoot growth from axillary meristems (Auldridge et al., 2006). Another significant member of this family is β-carotene 15, 15'-monooxygenase (BCO), formerly known as β-carotene 15,15'-dioxygenase, based on biochemical and amino acid sequence data (Lindqvist and Andersson, 2002; Moise et al., 2005). This enzyme shares 58% sequence coverage with CCD7 of Arabidopsis thaliana as established through BLASTp. BCO catalyzes the first step in the synthesis of retinol from dietary carotenoids. Retinol, also referred to as vitamin A, is a fat-soluble polyisoprenoid, in its various oxidative and isomeric forms is essential for embryonic development, pattern formation and vision. Because animals are unable to synthesize vitamin A de novo from endogenous isoprenoid precursors, they must instead derive it from cleavage of β-carotene and certain other carotenoids with an unsubstituted β-ring (e.g. γ- and α-carotenes, β-zeacarotene, and
β-carotene) (Redmond et al., 2001). Of the more than 600 different carotenoids isolated from nature, almost 50 possess biological activity; hence, these compounds are termed provitamin A carotenoids (Rock, 1997).

Retinol (Vitamin A) deficiency is known to be associated with calculus formation though mechanism involved is still unclear. In a study, the serum retinol level in the lithiatic patients was found to be low (Khanam et al., 1988). There are various probable mechanisms by which retinol deficiency may be involved in urolithiasis. The squamous metaplasia of the urinary tract can result in keratin debris which promotes calculus formation. Histological analysis showed squamous metaplasia that was confined to urinary tract (Munday et al., 2009). Vitamin A deficiency in children was seen to be associated with stone formation and was thought to be due to keratinisation of the urinary epithelium (Brown and Brown, 1941). Thus, there appears to be a relationship between urothelial changes in association with vitamin A deficiency which may lead to calculus formation. Vitamin A deficiency caused important changes in urine composition and there was a decrease in the concentration of urinary glycoaminoglycans and zinc. Lesions of cuboidal epithelia covering the papillae in rats treated with vitamin A deficient diet were severe. It was studied that in urolithiatic humans too, there is an increase in Vit E/Vit A ratio. These results could be related to the possible deficit of vitamin A in kidney of stone formers, this being one factor for urolith development. Moreover, deficit of important urinary crystallization inhibitors in stone formers (pyrophosphate and phytate) can also be related to presence of low levels of renal vitamin A which prevents enzymatic degradation of such inhibitors (Grases et al., 1998).
On amino acid analysis, TTP was found to be very rich in tyrosine. Literature suggests that presence of tyrosine in substrate binding cleft of BCO plays a crucial role in its catalytic activity by involvement in cationic intermediate stabilization, which plays an important role in the catalytic action of BCO. Site directed mutagenesis of these aromatic residues to leucine residues impair the catalytic activity (Poliakov et al., 2009). Tyrosine residue is also involved in the activity of the plant CCDs and is conserved amongst them (Snowden et al., 2005). Management of levels of retinol by the kidney is by retinol binding protein (RBP4), a protein which is found in the renal stone matrix and used as a marker for kidney function in chronic kidney disease (Henze et al., 2010). Since, RBP4 contains an unusually high content of tyrosine; it is possible that it play an important role in structure of the retinol-binding cleft, and in the interaction between retinol and RBP4 (Kanai et al., 1968). BCO leads to an increase in the amount of retinol by breakdown of β-carotene with the role of tyrosine in its catalytic action. Since, CCD7 belong to the same family as BCO and retinol happens to be a product of BCO which as cited in literature is known to possess antilithiatic potency, we hypothesize that in the light of above findings, even the product of CCD7 i.e. 10’ apo β-carotenal might be having a key role to play in preventing kidney stone formation (Figure 5.1).
TTP was found to be tyrosine rich.

**Role in BCO**
Tyrosine in substrate binding cleft of BCO plays a crucial role in its catalytic activity by involvement in cationic intermediate stabilization (Poleiskov et al., 2009).

**Role in RBP4**
Since RBP4, marker of kidney function in CKD, contains an unusually high content of aromatic amino acids (8 tyrosine residues per molecule), it is possible that the aromatic amino acid residues play an important role in structure of the retinol-binding cleft, and in the interaction between retinol and RBP4 (Kanai et al., 1998).

**Figure 3.1: Hypothesis about the role of CCD7 in urolithiasis**

CCD7 homologous to the purified protein from *Tribulus terrestris* (TTP) is also shown to contain an EF hand domain (228 – 240) which is a characteristic of Ca$^{2+}$ binding proteins. The name EF-hand was devised by Kretsinger and Nockolds over 25 years ago as a graphical description of the calcium-binding motif observed in parvalbumin. This structural motif has turned out to be very widespread, found in a large number of protein families: some 66 subfamilies are known to date (Lewis-Bentley A and Rety S, 2000).

The classical EF-hand is a helix-loop-helix motif characterized by a sequence of, usually, 12 residues with the pattern X•Y•Z•−Y•−X•−Z, where X, Y, Z, −X, −Y and −Z are the ligands that participate in metal coordination (Figure 4.30) and the dots represent intervening residues. At positions X and Y, we usually find the side chains of aspartic acid or asparagine; the side chains of aspartic acid, asparagine or serine are
found at Z and a peptide carbonyl oxygen lies at \(-Y\). \(-X\) is usually a water molecule and \(-Z\) is a conserved bidentate ligand, glutamic acid or aspartic acid. This sequence forms a loop that can accommodate calcium or magnesium with distinct geometries: magnesium is usually bound by six ligands in an octahedron, whereas seven ligands at the vertices of a pentagonal bipyramid coordinate calcium. In EF Hand Domain, helix E winds down the index finger, whereas helix F winds up the thumb of a right hand. When the calcium ion binds, helix F moves from the closed (apoprotein, light grey) to the open (holoprotein, dark grey) conformation. Examples of some EF-hand domain containing proteins are, the regulatory domain of scallop myosin, troponin C, parvalbumin, S100 proteins psoriasin and MRP8 (Lewit-Bentley A and Rety S, 2000). The calcium binding proteins that have been characterized by high-resolution x-ray crystal structure analysis fall into two general categories. One group includes many extracellular enzymes and proteins that have enhanced thermal stability or resistance to proteolytic degradation as a result of binding calcium ions. The other group is made up of a family of intracellular proteins that reversibly bind calcium ions. The second group is distinguished from the first in that its members have common calcium binding helix-loop-helix motif, termed an "EF-hand" that has been widely applied to describe calcium binding sites. It has been suggested by Mustafi and Nakagawa that most of kidney stone inhibitory proteins like nephrocalcin are similar to the proteins of the second group because they reversibly binds calcium ions (Mustafi and Nakagawa, 1994). In addition few known CaOx inhibitory proteins like osteonectin and calgranulin have also showed presence of calcium binding EF hand domains in them (Pillay et al., 1998).
We tested the protective potency of the purified protein towards oxalate induced cell injury on NRK-52E. It has also been proposed that exposure of renal epithelial cells to higher than normal levels of calcium and oxalate can perturb the plasma membrane, causing lateral and trans-membrane migration of phospholipids, sequestering them in specific domains. Migration of the acidic phospholipids such as phosphatidylserine from the inner leaflet of the plasma membrane to the outside promotes adhesion of CaOx crystals to the epithelial cells. Crystal attachment to the inner medullary collecting duct cells has also been correlated with membrane fluidity (Mandel, 1994). Recent studies have provided evidence that both Ox and CaOx crystals selectively activate p38 MAPK signal transduction pathways (Chaturvedi et al., 2002). In addition, p38 MAPK is essential for re-initiation of the induced DNA synthesis. Ox exposure also causes modest activation of JNK, as determined by c-Jun phosphorylation. Apparently the renal epithelial response to oxalate involves signal transduction via MAP kinases, similar to the cellular responses to many other challenges. Cytosolic phospholipase A₂ (cPLA₂) is released upon the activation of MAP kinases and translocated to the cell membrane. cPLA₂ preferentially hydrolyses arachidonoyl phospholipids generating a number of byproducts including arachidonic acid and lysophospholipids. Exposure of MDCK cells to oxalate produces a time and concentration dependent increase in cPLA₂ activity (Jonassen et al., 2003). Inhibition of cPLA₂ activity blocks the oxalate induced upregulation of Egr-1, c-jun and c-myc genes. An effective method of preventing crystal adherence is to neutralize potential binding sites at the cell surface or at the crystal surface, thus preventing the activation of various signal transduction pathways. Studies with cultured renal tubule cells have demonstrated that crystal binding can be inhibited by polyanionic molecules that are
naturally present in tubular fluid as they coat the crystalline surface. CaOx crystals rapidly adhere to anionic sites on the surface of cultured renal epithelial cells, but this process can be inhibited, if specific urinary anions such as glycosaminoglycans, uropontin, nephrocalcin, or citrate are available to coat the crystalline surface. Therefore, competition for the crystal surface between soluble anions in tubular fluid and anions on the apical cell surface could determine whether or not a crystal binds to the cell. Once bound, crystals are quickly internalized by renal cells; reorganization of the cytoskeleton, alterations in gene expression, and initiation of proliferation may then ensue (Leiske et al., 1995; 1999; Verkoelen et al., 1995). Over several weeks in culture, renal cells (BSC-1 line) dissolve internalized crystals, although once a cell binds a crystal, additional crystals are more likely to bind, possibly forming a positive feedback loop that results in kidney stone formation (Leiske et al., 1999). This may explain the protective role of the purified protein biomolecules from *T. terrestris* in increasing cell viability and decreasing percentage LDH release, which was more profound even at low concentration of 4μg/ml in comparison to 50μg/ml crude aqueous extract, as the purified protein is anionic in nature. This data further validates the high potency of the purified protein in playing a crucial role in protecting the renal epithelial cells against oxalate damage.