Renal stone disease has tormented people throughout the ages. Despite enormous developments in nephrology and urology, we still do not know exactly how kidney stones are formed and how to prevent them. Kidney stones are built from numerous tiny crystals that commonly are pasted together with organic material. The formation of crystals in the kidney is normal and harmless provided that they are excreted with the urine. The difference between stone formers and non–stone formers is that crystals stay behind in kidneys of stone formers. An acute renal colic that is caused by a tiny stone’s passing down the ureter can be extremely painful. Small stones (≤5 mm) often pass without medical intervention, but larger stones (>5 mm) usually must be removed. With the advent of extracorporeal shock wave lithotripsy and improved surgical procedures, the removal of kidney stones no longer is as primitive as a few hundred years ago. Nevertheless, recurrent stone disease causes significant morbidity, and in patients with inborn errors in endogenous oxalate synthesis, pathologic renal calcifications ultimately may destroy the kidney. The human kidney concentrates the urine to preserve water and essential nutrients and to eliminate waste products [274].

The formation of renal stones is a consequence of increased urinary supersaturation with subsequent formation of crystalline particles. Since most of the solid particles crystallizing within the urinary tract will be excreted freely, particle formation is by no means equivalent to symptomatic stone disease. However, when solid particles are retained within the kidney, they can grow to become full-size stones [11]. Crystals can be retained at many sites in the kidneys and undergo the size-enhancing process of growth and aggregation. In order for stones to be formed, not only do crystals need to be retained within the kidney, but they must be located at sites from which crystals can cause ulceration at the papillary surface to form a stone nidus. It is thought that renal tubular injury plays an important role at this point. Khan hypothesized that renal tubular injury promotes crystal retention and the development of a stone nidus on the renal papillary surface [12]. In addition, renal tubular injury enhances crystal nucleation at low supersaturation [157].
The process of attachment or endocytosis of crystals to renal tubular cells is what is generally meant by crystal–cell interactions. Crystal–cell interaction is the next step, and is also promoted by renal tubular injury. Since crystal formation is a common phenomenon in human urine and crystalluria per se is harmless, abnormal retention of formed particles must occur when kidney stones form. Thus, crystal–cell interactions may be highly relevant. The crystals that are internalized in the interstitium undergo growth and aggregation, and develop into renal stones.

Experimental induction of CaOx urolithiasis starts with hyperoxaluria followed by crystalluria and deposition in the kidney [12, 148]. Many studies have focused on oxalate-induced changes in renal epithelial cells. Recent studies indicated that oxalate-induced death of renal epithelial cells exhibits characteristics of both apoptotic and necrotic cell death [196]. Several studies have provided evidence that oxalate-induced toxicity is related to superoxide [217] and lipid peroxidation [191, 197]. The oxalate-induced increase in free radical production and cell death appear to be linked in a concentration-dependent manner. Oxalate also causes activation of at least two lipid signaling cascades, one involving phospholipase A2 (and resulting in increased release of arachidonic acid and formation of lysophospholipids [191] and the other involving sphingomyelinase (and resulting in increased levels of ceramide and decreased levels of sphingomyelin) [197]. Free radical mediators of inflammation have been shown to induce the production of chemokines and changes in gene expression [192].

Damaged or apoptotic renal cell monolayers present a number of binding sites for crystal attachment that are normally masked in intact epithelia, including sites enriched for PS, HA, sialic acid and/or matrix proteins [138]. Once attached, these crystals can serve as centers for the nucleation of new crystals, a process favoring stone development. Alternatively, crystals on the cell surface may be taken up by the renal cells (an active endocytic process) [146]. Once internalized, crystals may dissolve within lysosomes or re-emerge at the basolateral surface, providing centers for growth of stones in the renal interstitium [170]. Debris from damaged cells can also foster stone growth by providing centers for heterogeneous crystal nucleation. Human kidney stones contain an organic core whose composition resembles that of cellular membranes [275], and the addition of renal membrane fragments promotes heterogeneous nucleation of crystals in artificial urine [276].

It appears that the process of renal tubular cell injury is of key importance in renal stone formation. Though many aspects of the mechanism of renal stone formation remain
un unclear, it is certain that renal tubular cell injury is a very important part of it. At present, though few substances useful for the prevention of urolithiasis are available, the development of medications that prevent renal tubular cell injury will provide a novel strategy for preventing this disease [112].

Hence, this study was aimed to conduct the detailed studies to assess the effect *Terminalia arjuna* on the adhesion of COM crystals to cultured renal cells to establish a scientific basis for their anti-urolithiatic property and characterization of the antilithiatic proteins from *Terminalia arjuna*. Since, *Terminalia arjuna*, belonging to the family Combreaceae, holds a reputed position in Ayurvedic system of medicine since ancient times [18]. Experimental and clinical studies revealed the beneficial effects of this plant against all sorts of conditions of cardiac failure [19]. It was found to be efficient in management of kidney stones not only *in vitro* but *in vivo* too. *Terminalia arjuna* bark extract is previously reported to inhibit CaOx crystal precipitation and growth [277]. Hence, characterization of antilithiatic proteins from *Terminalia arjuna* can open new vistas for the therapeutic proteins present in *Terminalia arjuna* for the treatment and cure of urolithiasis.

### 5.1 Antiurolithiatic potential of aqueous extract of *Terminalia arjuna* *in vitro*

In the present study, the inhibitory potential of *Terminalia arjuna* was evaluated *in vitro* on calcium oxalate crystallization and crystal growth. 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 [278]. The effect of *Terminalia arjuna* bark aqueous extract on CaOx crystallization and crystal growth kinetics was studied by the time course measurement of turbidity. With respect to calcium oxalate crystallization, the aqueous extract of the plant was effective in inhibiting both the nucleation and aggregation of CaOx crystals in a concentration-dependent manner. The aqueous extract of the plant also inhibited the CaOx crystal growth effectively. Moreover, a study was performed to evaluate the crystal dissolution potential of aqueous extract of *Terminalia arjuna* bark against CaOx monohydrate crystals. In the microscopic study, aqueous extract modified CaOx monohydrate crystal morphology. A similar change in the morphology of CaOx monohydrate crystals has been previously reported with citrate and Mg$^{2+}$ [279]. Microphotography studies verified that aqueous extract of *Terminalia arjuna* resulted
in the formation of round CaOx crystals. COM and COD are the major forms found in most urinary calculi. Aqueous extract of *Terminalia arjuna* inhibited the growth of COM crystals, prevented the aggregation of COM crystals, and induced the formation of spherical COM crystals. These spherical COM crystals are thermodynamically less stable phase and have weaker affinity for cell membranes than hexagonal COM crystals. Both the positive control cystone drug (1000 μg/mL) and aqueous extract of *Terminalia arjuna* (1000 μg/mL) modified the morphology of hexagonal CaOx monohydrate crystals to more rounded edges and spherical shape, as shown in Figure 4.3B and 4.3C respectively. This shape may prevent the formation of kidney stones, because crystals with this shape are more easily excreted in the urine compared with the COM. Furthermore, *Terminalia arjuna* bark aqueous extract possess very high antioxidant activity due to the presence of Terpenoids. It has the ability to scavenge the free radicals with an IC$_{50}$ of 13.11 μg/mL. Thus, pretreatment with antioxidants can block oxalate induced increases in ceramide [197]. Antioxidant treatments also block oxalate-induced cell death [211], suggesting a role for oxidant stress in these responses.

5.2 Diminution of oxalate-induced renal tubular epithelial cell injury by aqueous extract of *Terminalia arjuna*

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 [280]. Since, it is known that hyperoxaluria is a major risk factor for CaOx nephrolithiasis, exposure to high levels of oxalate and/or COM crystals is injurious to renal epithelial cells and triggers serial responses related to stone formation. High level of oxalate causes 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 either by apoptosis or necrosis. These changes are significant predisposing factors for the facilitation of crystal adherence and retention [12, 196]. The duration of exposure and concentration of oxalate to which cells were exposed were selected based on results of earlier studies and the likelihood of occurrence inside the kidneys. The concentration of oxalate in the urine changes as it moves through the nephron and is 0.22 mM in normal excreted urine, 0.44 mM in
conditions of mild hyperoxaluria and 1.5 mM in primary hyperoxaluria. Various studies have used 0.1–4 mM oxalate [281] for exposure of renal epithelial cells in vitro.

In the in vitro cell culture study with NRK-52E and MDCK cells, *Terminalia arjuna* proved to have a protective effect towards the oxalate induced renal epithelial cell injury. When NRK-52E and MDCK cells were injured by exposure to 2 mM oxalate for 48 hours, the aqueous extract reduced the injury in a dose-dependent manner as verified by trypan blue exclusion and MTT viability assays. The adhesion and internalization of CaOx crystals to the cell surface of NRK-52E and MDCK was also studied in the absence and presence of aqueous extract of *Terminalia arjuna*. When the cells were exposed to oxalate, some of the CaOx crystals adhered tightly and some internalized into renal cells with subsequent detrimental effects to the cells [260]. These crystals caused cell damage and cell death either by apoptosis or necrosis which is evident by lesser number of cells. The aqueous extract reduced the injury to the renal cells by disrupting the interaction of CaOx crystals with the cells which was evident by increase in the number of viable cells. A study suggested that the adhesion of the radioactive CaOx crystals to the Madin Darby canine kidney (MDCK) cells was inhibited in the presence of the aqueous extract of *Herniaria hirsuta* in a concentration dependent manner [282].

We demonstrated that exposure to 2 mM oxalate for 48 hours caused induction of apoptosis leading to increased cell death. The morphological changes in cell nuclei were determined by fluorescence microscopy by staining cells with Hoechst 33258 dye. The cells treated with oxalate showed marked changes in morphology such as irregular shape, membrane blebbing, apoptotic bodies and condensed and fragmented chromatin. The aqueous extract exhibited cytoprotective potential which was apparent by larger number of viable cells with intact cellular membrane and fewer apoptotic bodies, showing reduced level of apoptosis. This was further confirmed by flow cytometry analysis using Annexin V/PI staining and Anti-Active Caspase-3 antibody staining. When renal cells were injured with oxalate and/or CaOx crystals, the number of cells that bind to annexin V increased, indicating that phosphatidylserine is exposed on the cell luminal surface. This observation is not only consistent with literature indicating that exposure to high concentration of oxalate results in the relocation of anionic phospholipids that are normally confined to the inner leaflet of the plasma membrane, but also firmly establishes the role of phosphatidylserine in crystal attachment. The
treatment with aqueous extract significantly reduced the number of apoptotic cells by disrupting the interaction of CaOx crystals with the cells.

In a recent study, it has also been shown that oxalate induces the exposure of phosphatidylserine on the surface of IMCD cells in culture and the exposure was temporal to the attachment of CaOx crystals to the cells [163]. Exposure to high levels of oxalate in vitro [196] and in vivo [203] leads to an increase in the abundance of apoptotic renal epithelial cells by a process involving increased oxidant stress [196]. ROS generated caused perturbations in mitochondrial function are often accompanied by an increase in mitochondrial permeability and a release of pro-apoptotic factors. These factors in turn trigger the activation of cellular caspases, serine proteases that have been linked to apoptotic cell death [204, 226]. Caspase-3 is a key protease that is activated during the early stages of apoptosis. We confirmed that when renal cells exposed to oxalate, the number of apoptotic cells significantly increased which were detected by anti-active caspase-3 antibody. The addition of aqueous extract reduced the number of apoptotic cells thus proving its ability to protect against oxalate induced renal cell injury. Our recent findings provide a possible explanation for these findings, e.g. oxalate actions at the cell membrane generate lipid signals that act on mitochondria to elicit an increase in oxidant stress and an increase in apoptotic death [197].

These data clearly indicate that oxalate and/or CaOx crystals are toxic to renal tubular epithelial cells. We postulated that the aqueous extract of Terminalia arjuna may contain substances that inhibit CaOx crystallization and crystal growth kinetics. Binding of the CaOx crystals to the renal epithelial surface and/or interaction of oxalate ions with calcium ions are blocked by active biomolecules of the plants [282]. Thus, Terminalia arjuna exhibited renoprotective role towards the oxalate induced cell injury. The mechanism of inhibition /reduction in the injury needs to be studied further. Hence, characterization of antilithiatic proteins from Terminalia arjuna can open new vistas for the therapeutic proteins present in Terminalia arjuna for the treatment and cure of urolithiasis.

5.3 Purification and characterization of antilithiatic proteins from Terminalia arjuna

Proteins were extracted from the bark of Terminalia arjuna to investigate the antilithiatic activity of plant proteins involved in the inhibition of calcium oxalate stone formation process. Protein estimation of the whole protein extract, separated into <3
kDa and >3 kDa fractions was done by Bradford assay. It was found that the protein concentration of the whole extract was 345.11 μg/mL, while that of <3 kDa and >3 kDa was 68.58 μg/mL and 273.44 μg/mL respectively. The bioactivity of whole protein extract, <3 kDa and >3 kDa fractions was investigated through CaOx crystallization and crystal growth assay systems. The whole extract possessed inhibitory activity against CaOx crystal nucleation, aggregation and growth kinetics. The <3 kDa fraction showed inhibitory activity towards CaOx crystal nucleation, aggregation and crystal growth assay system which was less than whole protein extract. The >3 kDa fraction showed inhibitory activity towards CaOx crystal nucleation, aggregation and growth assay system which was more than whole protein extract. SDS-PAGE of >3 kDa proteins suggested that large number of proteins are present in the bark of *Terminalia arjuna*. A total of 16 bands were detected by silver staining. Based on the above observations, the >3 kDa fraction was thus chosen for further investigation of its bioactivity against oxalate injured NRK-52E and MDCK renal epithelial cells. More than 3 kDa fraction exhibiting highest activity on CaOx crystal crystallization and growth assay was subjected to chromatography to purify proteins. The purification was performed systematically using anion exchange and molecular-sieve chromatography followed by bioactivity testing against CaOx crystal crystallization and growth assay and SDS-PAGE analysis after each purification step. After conducting anion exchange chromatography it was found that the eluted peaks P1, P2 and P3 exhibited inhibitory activity against CaOx crystal nucleation, aggregation and growth kinetics system. SDS-PAGE analysis showed presence of few bands in each peak. These peaks P1, P2 and P3 were then further purified individually by molecular-sieve chromatography, the purified proteins obtained from each peak were tested for their bioactivity against CaOx crystal crystallization and growth. The most potent fraction A1 obtained from purification of P1 possessed inhibitory activity against both CaOx crystal nucleation, aggregation and growth assay system. B1 and B2 were the most potent fractions obtained from purification of P2. B1 and B2 possessed inhibitory activity against CaOx crystal nucleation, aggregation and growth systems. The most potent fractions C1 obtained from purification of P3 possessed inhibitory activity against both CaOx crystal nucleation, aggregation and growth. The purified potent fractions A1, B1, B2 and C1 showed the presence of multiple bands when analyzed by 12% SDS-PAGE, but showed the presence of single bands of MW ~190 kDa, ~130 kDa, ~90 kDa and ~90 kDa respectively when further analyzed by 12% NATIVE-
The homogeneity of the purified fractions was confirmed on RP-HPLC which showed a single peak. When the effect of these purified proteins was tested on oxalate injured NRK-52E and MDCK renal epithelial cells for their activity, it was found that A1, B1, B2 and C1 diminished the cellular injury caused by oxalate. Analysis of fractions A1, B1, B2 and C1 by MALDI-TOF-MS resulted in peptide mass fingerprint which when followed by database search on a MASCOT server matched significantly with Nuclear pore anchor, DEAD Box ATP-dependent RNA helicase 45, Lon protease homolog 1 and Heat shock protein 90-3. Our finding suggest presence of the above mentioned, as anionic proteins present in the bark of *Terminalia arjuna* with capability to modulate CaOx crystallization.

The identified Nuclear pore anchor (NUA) is an inhibitor protein which inhibits calcium oxalate crystal growth. It is a 237 kDa protein encoded by the gene AT1G79280.2 and is localized to the inner surface of the nuclear envelope and is a component of the nuclear pore. The nuclear pore complex (NPC) is a large multiprotein complex that is the sole gateway of macromolecular trafficking between the cytoplasm and the nucleus [266]. Nuclear membrane has oxalate binding at pH 7.4. The oxalate binding protein plays a vital role in the transport of oxalate. Oxalate transport across cellular membranes is mediated by anion-exchange transport proteins. Under physiological conditions, the NPC is an inhibitor of CaOx crystal nucleation, aggregation and growth. The expression of NPC increases in nephrolithiasis [267]. Also, it was observed that this protein has Lysine and glutamic acid rich region. Basic amino acid lysine is involved in oxalate binding which is evident by the fact that all oxalate binding proteins were sensitive to the transport inhibitor 4’-4’ diisothiocyanostilbene-2–2 disulphonic acid (DIDS), which is known to interact with the lysine moiety of the proteins. Now there is an evidence of oxalate specific binding molecules with similar crystal growth modulating activity. They occur in the renal medulla and cortex and are differentially abundant in the subcellular organelles. All the oxalate binding proteins possess lysine in the active binding site. Lysine modification abolishes oxalate-binding activity [267]. In addition to lysine rich region, the presence of acidic polyglutamic acid residues may bind to calcium ions and thus, preventing the adhesion of COM crystals to the epithelial cell surface. Since COM and hydroxyapatite crystals adhered to negatively charged cell surface molecules by a process that could be inhibited by GAG, polyglutamic acid, polyaspartic acid, nephrocalcin, uropontin, and
citrate but not by THP. Anions that inhibited adhesion of crystals appeared to act on the crystal surface [149]. Hence, the exogenous supply of NUA protein may play a vital role in inhibiting the CaOx crystallization.

The second protein is a DEAD Box ATP-dependent RNA helicase 45. RNA helicases are prominent candidates as RNA chaperones because the energy derived from ATP hydrolysis can be used to promote the formation of optimal RNA structures via local RNA unwinding, or by mediating RNA–protein association/dissociation [268]. Helicases include six superfamilies (SF1 to SF6). The majority of known RNA helicases belong to the SF2 superfamily, which can be subdivided into several families including DEAD, DEAH and DExH/D. DEAD-box proteins comprise the largest and most extensively characterized family of RNA helicases. These proteins are characterized by a core of 350 to 400 amino acids containing seven to nine conserved amino acid motifs. In the DEAD-box proteins, motif II includes the sequence D-E-A-D (Asp-Glu-Ala-Asp), from which the name was derived [269]. Recent advances suggested some molecular mechanisms that enable DEAD/H RNA helicases to sustain cell survival, coordinate stress responses, and mediate cell death, by microbial pathogen-induced signaling cascades and stress granule formation triggered by various stress stimuli. Cell survival mechanism of Stress Granules is linked to reactive oxygen species (ROS) production. A recent study reported that Stress Granules harbor antioxidant activity, partly mediated by two Stress Granules components, G3BP1 (GTPase-activating protein SH3 domain binding protein 1) and USP10 (ubiquitin-specific protease 10). USP10 possesses an antioxidant activity. However, under steady-state conditions, its activity is suppressed by excess G3BP1. Upon stress, G3BP1 and USP10 cooperatively induce Stress Granules. Meanwhile, Stress Granules disrupt G3BP1-mediated inhibition against USP10, possibly by altering the conformation of USP10 and/or G3BP1, thereby uncovering the antioxidant activity of USP10 to reduce ROS production. The authors proposed that Stress Granules may act as rapidly inducible antioxidant machinery protecting cells from ROS-induced apoptosis [269]. The role of Stress Granules in controlling apoptosis could be linked to sequestration of apoptosis-promoting factors, suppression of ROS production and reprogramming of mRNA expression upon stress [269]. Since, this protein is rich in glutamic acid and aspartic acid amino acids, this protein may bind to calcium ions and thus, preventing the adhesion of COM crystals to the epithelial cell surface. Since COM and hydroxyapatite crystals adhered to negatively charged cell surface molecules by a
process that could be inhibited by GAG, polyglutamic acid, polyaspartic acid, nephrocalcin, uropontin, and citrate but not by THP. Anions that inhibited adhesion of crystals appeared to act on the crystal surface [149].

The third protein which is Lon protease homolog 1, is an enzyme that in humans is encoded by the LONP1 gene. This gene encoded a mitochondrial matrix protein that is the subunit of a barrel-shaped homo-oligometric protein complex, the Lon protease. In mitochondrial matrix, a majority of damaged proteins are removed via proteolysis led by Lon protease, which is an essential mechanism for mitochondrial protein quality control (PQC). This protein contains Arg-Gly-Asp (RGD) tripeptide, a cell attachment sequence which binds to integrin [270]. Proteins that contain the RGD attachment site, together with the integrins that serve as receptors for them, constitute a major recognition system for cell adhesion [270]. RGD was originally identified as the sequence in fibronectin that engages the fibronectin receptor, integrin α5β1. Although RGD peptides inhibit ligand binding to integrin with an RGD recognition specificity [283]. Adhesion of urinary crystals to the apical surface of renal tubular cells could be a critical step in the formation of kidney stones. The negative regulators of CaOx crystal uptake include heparin, transforming growth factor-β2 (TGF-β2), and the tetrapeptide arginine-lycine aspartic acid-serine (RGDS) [146]. Studies by Lieske and colleagues demonstrated an attenuation of crystal attachment in BSC-1 cells by treatment with arginine-glycine-aspartic acid-serine (RGDS, a tetrapeptide that bind to integrin), or by pretreatment with fibronectin, a connective tissue protein containing this peptide sequence [141, 146]. Crystal endocytosis can be inhibited by agents that interact with cell adhesion sites including RGDS, fibronectin and heparin. The degree of CaOx crystal deposition was inhibited by 60-80% in the cyclic RGD pretreated MDCK cells [146].

The fourth protein identified as Heat shock protein 90-3 is a molecular chaperone possessing antiapoptotic activity. Hsp90 is responsible for the refolding of denatured proteins as well as the three dimensional maturation and transport of more than 200 client proteins [271]. Heat-shock proteins (HSPs) are a highly conserved family of molecular chaperones, some of which are induced by sublethal cellular stresses, including temperature elevation, hypoxia and oxidative damage. Heat-shock proteins negatively regulate apoptosis [272]. Heat-shock protein 90 promotes cell survival by activation of NF-κB. Tumour necrosis factor-α activation recruits and stabilises receptor interacting protein (RIP) at the TNF receptor-1 to maintain NFκB activity. Key
signalling molecules, such as VEGF, induces antiapoptotic protein Bcl-2 expression and stimulates HSP90 association with Bcl-2 and Apaf-1 to inhibit apoptosis [273]. Also, Heat shock protein 90-3 has a glutamic rich region. The presence of acidic polyglutamic acid residues may bind to calcium ions and thus, preventing the adhesion of COM crystals to the epithelial cell surface. Since COM and hydroxyapatite crystals adhered to negatively charged cell surface molecules by a process that could be inhibited by GAG, polyglutamic acid, polyaspartic acid, nephrocalcin, uropontin, and citrate but not by THP. Anions that inhibited adhesion of crystals appeared to act on the crystal surface [149].

The working hypothesis that was confirmed in this study is that oxalate and/or COM crystals induce oxidative stress that contributes to renal tubular epithelial cell injury followed by death either by apoptosis and/or necrosis. Most of the cells underwent apoptotic cell death and few cells underwent necrotic cell death, may be in response to alterations in mitochondrial function that are characterized by initial increase in free-radical production, followed by a dissipation of the mitochondrial membrane potential and release of proapoptotic factors. Apoptosis of renal tubular cells in response to oxalate and CaOx crystals may play a significant role in CaOx urolithiasis. Apoptotic changes include exposure of annexin binding phosphatidylserine to the cell surface. Clusters of negatively charged head groups of phosphatidylserine attract calcium and can act as sites for attachment of calcific crystals to cell surfaces [284]. Such clusters on the surface of apoptotic bodies and membranous cellular degradation products can promote crystal nucleation [285]. Exposure of the basement membrane after detachment of cells or cellular debris, promoting calcium oxalate crystal adhesion and thus favoring crystal retention and lithogenesis, important in the pathogenesis of urolithiasis.

When renal epithelial cells are exposed to oxalate ions and CaOx crystals, there is an increase in expression of immediate early genes (c-myc, Egr-1, c-jun and nur-77) and production of several urinary macromolecules (Tamm-Horsfall protein, Osteopontin, Prothrombin fragment-1, Bikunin and inter-α-inhibitor, α1-Microglobulin, CD44, Calgranulin, Heparan sulfate, Osteonectin, Fibronectin, Matrix Gla Protein), which modulate the nucleation, growth, aggregation and retention of crystals in the kidneys [286]. The calcium binding property of these molecules enables them to interact with calcium containing crystals. Some of them, such as OPN, have specific domains to interact with cell membranes, which facilitate their immobilization and promotion of
crystal attachment. Almost all of the modulators are produced by the kidneys and excreted in the urine [222, 223].

In the present study Nuclear pore anchor, DEAD Box ATP-dependent RNA helicase 45, Lon protease homolog 1 and Heat shock protein 90-3 were identified as anionic inhibitors of CaOx crystallization from the bark of *Terminalia arjuna*. We observed that these anionic proteins possessed antilithiatic activity which was evident by the inhibition of CaOx crystallization and crystal growth kinetics. Also, these proteins protected the renal epithelial cells NRK-52E and MDCK from oxalate induced injury. Since, these proteins contain either polyglutamic acid, polyaspartic acid, polylysine rich regions and/or RGD sequence. Some of them also have anti-apoptotic activity and stimulates cell survival by inhibiting the proapoptotic factors.

Urinary Trefoil Factor 1 is an inhibitor of CaOx crystal growth. The 4C-terminal glutamic residues of TFF1 interact with calcium ions to prevent CaOx crystal growth [287]. The Fibronectin, a connective tissue protein containing this tripeptide sequence, is known as an inhibitor of CaOx [141, 146]. It has also been reported that FN protected against renal tubular cell injury caused by oxalate and COM crystals. The Osteopontin also containing this tripeptide sequence and rich in glutamic acid, arginine residues, inhibits nucleation, aggregation, growth and cellular attachment of CaOx crystals [288]. We postulated that exogenous supply of Nuclear pore anchor, DEAD Box ATP-dependent RNA helicase 45, Lon protease homolog 1 and Heat shock protein 90-3 proteins may play a vital role in inhibiting the CaOx crystallization and open new vistas to study therapeutic proteins from plants for the treatment of urolithiasis.