CHAPTER 6
CONCLUSION

Nephrolithiasis, or kidney stone, is the presence of renal calculi caused by a disruption in the balance between solubility and precipitation of salts in the urinary tract and in the kidneys. Kidney stones develop when urine becomes “supersaturated” with insoluble compounds containing calcium, oxalate (CaOx), and phosphate (CaP), resulting from dehydration or a genetic predisposition to over-excrete these ions in the urine. Approximately 70-80% of kidney stones are composed of calcium either in pure form or mixed with a majority of pure CaOx stones. CaOx kidney stones are found in two different varieties, COM or Whewellite, and COD or Weddellite. COM, the thermodynamically most stable form, is observed more frequently in clinical stones than COD and it has greater affinity for renal tubular cells, thus responsible for the formation of stones in kidney.

The problem of stone formation produces pain and obstruct the flow of urine as the stones formed are unable to travel through ureter. It also causes severe back ache (the worst pain known as colicky pain is produced in the lower back), bloody, cloudy, and smelly urine, sickness, urge for urination, burning sensation during urination, fever, chills etc., less urine volume, change in urinary pH, and infections [289]. Available standard pharmaceutical drugs used in preventing and curing renal calculi are not effective in all patients and may produce adverse effects on long term use [290]. The treatment of urolithiasis is mainly considered with the dissolution of existing stones and preventing the reoccurrence of stones. Standard pharmaceutical drugs used to prevent and cure urolithiasis are not effective in all cases. Surgical treatment causes some problems like long term renal damage, hypertension and reoccurrence of stones. Extracorporeal shock wave lithotripsy is considered as a revolution in treating renal stones, but this treatment also causes some problems like long term renal damage, hypertension and reoccurrence of stones [291].

Scientific studies are mostly focused on phytotherapy as it is proved to be vital in preventing reoccurrence of stones [292]. Herbal drugs are reported to be effective with no side effects. The drug for prevention of the disease or its reoccurrence is of great interest as no drug in clinical therapy is of satisfactory result [293]. Herbal agents act
by allowing spontaneous passage of small calculi in urine by increasing the urinary volume and pH. The herbs also act by regulating oxalate metabolism, by maintaining balance between inhibitors and promoters of crystallization, by producing anti-oxidant, anti-microbial, analgesic, anti-inflammatory activities [294]. Modern medicine are proved to target only one aspect of urolithiatic pathophysiology whereas herbal remedies have been shown to exert effectiveness at different stages of stone pathophysiology.

In the present research, in vitro antilithiatic properties of *Terminalia arjuna* have been evaluated. The bark of *Terminalia arjuna*, locally named as “Arjuna” in India, is known in Ayurveda for the treatment of cardiovascular disorders. Till date, various plant extracts have been studied to reduce the incidence of calcium stone deposition both in vivo and in vitro [282, 295, 296] but the identification of naturally occurring CaOx inhibitory biomolecules from plants was hampered in past by limitation in identification method. Initially in vitro antilithiatic potential of aqueous extract of *Terminalia arjuna* was assessed and its cytoprotective role was evaluated on oxalate induced renal tubular epithelial cell injury. Further, from the bark of *Terminalia arjuna* antilithiatic proteins were isolated and characterized. Finally, the efficacy of these purified proteins was evaluated on oxalate injured renal tubular epithelial cell lines NRK-52E and MDCK. The conclusions made from results obtained at every step of the study are summarized point wise.

1. *Terminalia arjuna* aqueous extract exhibited a concentration dependent inhibition of CaOx crystal nucleation and aggregation. The extract also inhibited the growth of CaOx crystals effectively. Moreover, the aqueous extract changed the morphology of COM crystals from hexagonal shape to spherical form with rounded edges which have weaker affinity for cell membranes than hexagonal COM crystals. Aqueous extract of *Terminalia arjuna* also possessed antilithiatic activity which may protect the renal cells from oxalate induced oxidative stress.

2. Reduction of oxalate induced injury by aqueous extracts of *Terminalia arjuna* was evaluated on NRK-52E and MDCK cells w.r.t. cell viability, CaOx crystal adherence and apoptotic changes wherein the extract of the plant protected renal cells from the injury caused by oxalate in a dose dependent manner. The antioxidant potential of *Terminalia arjuna* may contribute to the decrease in the free radicals
released due to injury caused by oxalate thus suggesting the protective potential of *Terminalia arjuna*. The plant may also coat the cells thereby blocking the interaction of the crystals with the renal epithelium thus preventing their adherence and further agglomeration.

3. Proteins were extracted from the bark of *Terminalia arjuna* and the bioactivity of whole protein extract, <3 kDa and >3 kDa fractions was investigated by CaOx crystallization and crystal growth kinetics 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 suggests that plentiful proteins are present in the bark of *Terminalia arjuna*. A total of 16 bands were detected which are of both high and low molecular weight. Bioactivity of the >3 kDa fraction was investigated against oxalate injured NRK-52E and MDCK cells. >3 kDa fraction reflected inhibitory activity thereby leading to a decreased cell death in a dose dependent manner.

4. A total of four anionic inhibitors of CaOx crystallization were purified and identified from bark of *Terminalia arjuna* by bioactivity guided purification using anion exchange chromatography and molecular sieve chromatography along with validation with SDS-PAGE and Native-PAGE analysis. Finally, homogeneity of purified protein was confirmed by RP-HPLC. Further, the protein was in gel tryptic digested and characterized by MALDI-TOF-MS. Proteins were identified as Nuclear pore anchor, DEAD Box ATP-dependent RNA helicase 45, Lon protease homolog 1 and Heat shock protein 90-3 when m/z data obtained after MALDI-TOF-MS of digested protein was searched in MASCOT search engine. Proteins present in *Terminalia arjuna* were found to have an ability to inhibit CaOx crystallization and crystal growth it was observed that this inhibitory ability increased with successive protein purification steps.
5. Purified proteins from *Terminalia arjuna* also exhibited cytoprotective effect on NRK-52E and MDCK cells w.r.t. cell viability, CaOx crystal adherence to cells and apoptotic changes which was much more in comparison to the cytoprotective potential by aqueous extract. The protective potential of purified proteins from the bark of *Terminalia arjuna* in protecting renal cells from oxalate induced injury and cell death by apoptosis, thereby increasing the cell viability, was more profound even at a concentration of 10 µg/mL in comparison to 40 µg/mL of aqueous extract.

These results suggest that the purified proteins are inhibitors of CaOx crystallization and crystal growth and reduce the oxalate-induced renal cellular injury, thereby leading to enhanced cell viability. Polyanionic compounds coat the crystalline surface thus, inhibiting the binding of the crystals to the cells. The presence of specific cell attachment sequence also prevents the interaction and adhesion of CaOx crystals to the negatively charged head groups exposed on the cell surface as a result of oxalate exposure to renal cells. The purified proteins from the bark of *Terminalia arjuna* were anionic in nature due to presence of glutamic acid rich region and aspartic acid rich region and possessed cell attachment sequence (RGD, tripeptide that binds to integrins) thus, explaining the protective potential by increasing the cell viability, loss of CaOx crystal adherence to cells and reducing the induction of apoptosis in their presence. Our present results corroborate that this indigenous plant can be successfully used as an alternative treatment for urolithiasis. The data provides a rationale for the use of plant proteins as therapeutic agents to treat urolithiasis. The work presented here will open new vistas for protein therapeutics from medicinal plants for the management of urolithiasis.