Chapter 5

Discussion

Kidney stone formation or urolithiasis is a complex process that results from a succession of several physico-chemical events including supersaturation, nucleation, growth, aggregation, and retention within renal tubules [100]. It is a multi factorial disorder resulting from metabolic abnormalities influencing the composition of body fluids and urine. It affects about 1 – 3% of the population and the recurrence rate is quite high, about 50% at 10 years and 75% at 15 if untreated [101]. Several factors such as heredity, age and sex, geographical factors, climate, race, and diet have been suggested for the etiology of stone disease. The major risk factors for recurrences are suggested to be male sex, multiple and lower calyx stones, early onset, familial history, and complications after stone removal [102].

The majority of upper urinary stones are composed of calcium oxalate and calcium phosphate and usually occur in men, while most stones of magnesium and ammonium phosphate occur in the bladder, mostly in women [103]. Kidney stones often occur when urine becomes too concentrated [7]. This causes calcium, oxalate and phosphate or other chemicals in the urine to form crystals
on the inner surfaces of kidneys. This stage is called as initial mineral phase formation. Over the time, these crystals may combine to form a small, hard mass called as stones and stage is referred to as subsequent growth of crystals. Besides this, an uneven balance of acid in the urine and lack of substances in the urine that prevent the growth of crystals also affects the ability of stone forming substances to remain dissolved.

Urinary calculi are the major prevalent disorder in the urinary system [104]. Epidemiological data collected during several decades showed that the majority of stones, approximately, 80% are composed of calcium oxalate (CaOx) and calcium phosphate (CaP) [105]. Urinary calculi may cause obstruction, hydronephrosis, infection, and hemorrhage in the urinary tract system. It is widely known that urolithiasis is characterized by high recurrence if patients are not treated appropriately. Among the treatments used are extracorporeal shock wave lithotripsy (ESWL) and drug treatment. Besides high treatment cost and the exposure to shock waves in therapeutic doses may cause acute renal injury, decrease in renal function, and an increase in stone recurrence [106, 107]. In addition, persistent residual stone fragments and possibility of infection after ESWL pose a serious problem in the treatment of stones. Also, even though drug treatment has shown some feasibility in many randomized trials, it is not accomplished without side effects, which are severe at times [108, 109]. Therefore, it is worthwhile to look for an alternative therapeutic method by using medicinal plants or phytotherapy. Indeed, herbal medicine is as ancient as the history of mankind. Keeping this in mind, a number of plants are being studied all over the world for their medicinal values. Many of them have been classified as antilithiatic plants. Recent studies conducted in our lab on medicinal plants like Trachyspermum ammi [110], Dolichos biflorus (L.) [111] and Tribulus terrestris [112] showed their antilithiatic properties. In the present study au-
tillithtic behavior of *Tamarindus indica* (fruit) and *Terminalia arjuna* (bark) has been studied both *in vitro* and *in vivo*. First, the homogenous systems of *in vitro* mineralization were employed under physiological conditions of temperature, pH and ionic strength of the media to see the effect of *Tamarindus indica* and *Terminalia arjuna* on CaP initial mineral phase formation, its subsequent growth and demineralization and on growth of CaOx crystals. Both the plants were found to be inhibitory against urolithiasis.

**In vitro and in vivo efficacy of Tamarindus indica**

*Tamarindus indica*, commonly known as tamarind is well known for its medicinal values. The seeds of the plant inhibit the growth of urinary crystals and are used in the treatment of recurrent kidney stones in patients [113]. The effect of ingestion of 3 and 10 g of tamarind pulp (*Tamarindus indicus*) was studied in normal subjects and in stone formers. Tamarind intake at the dose of 10 g showed significant beneficial effect in inhibiting spontaneous crystallization in both normal subjects and in stone formers [57]. In another research it was observed that fewer occurrences of urinary calculi are found in southern India, which may be due to regular dietary intake of tamarind [114]. Another study group found that the inhibition of calcium hydrogen phosphate dehydrate crystals increases as the concentration of tartaric acid increases; consequently, the number of grown crystals and their average size decreases and hence it was concluded that tartaric acid inhibits the growth of CHPD crystals *in vitro* [3].

*Tamarindus indica* exhibited inhibition against various stages of stone formation. It showed inhibition against initial mineral phase formation, its subsequent growth and and demineralization of CaP and against growth of CaOx crystals *in vitro*. After confirming the antilithiatic nature *in vitro*, further validation was done *in vivo* on experimentally induced hyperoxaluric rat models. The rats induced with EG and NH4Cl showed a marked elevation of urinary injury marker
CHAPTER 5. DISCUSSION

enzymes (LDH and AP). This suggests damage to the brush border membrane of renal tubule, which appears to be associated with the retention and deposition of crystals in the kidney [115]. This excretion was normalized by putting animals on plant dose clearly showing antilithiatic properties of *Tamarindus indica* fruit. Further, creatinine clearance was observed, which is a useful measure for determining renal functioning. The impairment of renal functioning after treating animals with EG and *NH₄Cl* is an outcome of calcium oxalate crystals deposition in renal tissue. The effect of EG and *NH₄Cl* on renal functioning has been studied by many study groups [116]. This impairment was ameliorated when 5% and 10% of crude aqueous extract of the plant was given to the animals. Improvement in renal injury was well supported by histopathological studies. Decreased renal injury decreases sites for calcium oxalate deposition. Administration of crude plant extract to hyperoxaluric rats, prevents supersaturation of calcium oxalate and thus decreased their deposition in renal tubules.

Citrate and tartrate are known as good inhibitors of kidney stones [117]. In *Tamarindus indica*, both of them are present, which may contribute to its antilithiatic properties [118]. Studies have been done showing tartaric acid as inhibitor of CaP stones. Tartrates are expected to form metal ion complexes with calcium. Tartrates bind with the cations needed for crystal formation and subsequent growth, and also function as a crystal growth inhibitor of calcium oxalate by chemical adsorption on the crystallization sites at a growing interface [119]. An evaluation of tamarind and tartaric acid as inhibitor of calcium oxalate crystallization in urine is also reported [120]. Isolation and purification of the biomolecule(s) present in the aqueous extract of *Tamarindus indica* which was responsible for the antilithiatic properties was started with ultrafiltration. Crude aqueous extract was separated into two fractions, one having molecular weight less than 10 kDa and another having molecular weight greater than
10 kDa. Greater than 10 kDa fraction showed better inhibition and was further purified by applying anion exchange chromatography and molecular sieve chromatography. Protein fraction so obtained was loaded in SDS-PAGE and an anionic protein band having molecular weight of \( \sim 97 \) kDa was seen. Further, it has been seen that out of all the biomolecules present in *Tamarindus indica*, proteins make up nearly 8% [121]. Medicinal plants viz. *Trachyspermum ammi* [110] and *Dolichos biflorus (L.*)* [111] are also shown to have antilithiatic proteins in them.

**In vitro and in vivo efficacy of Terminalia arjuna**

Studies conducted on second medicinal plant, *Terminalia arjuna* also showed inhibition against CaP and CaOx crystallization. Bark of *Terminalia arjuna* is known to possess diuretic properties [122]. Because of its diuretic action, it is helpful for renal or urinary bladder stones. It is very helpful in polyurea condition and is also helpful in regularizing the increased urine frequency. It helps to tone up the urinary tract thus reduces the chances of formation of nidus in the tract, as the site of nidus formation is considered as the kidney stone deposition point. It is even effective in the building up the resistance against infection in the kidneys. A research conducted on BHUX, a patented polyherbal formulation consisting of the aqueous fraction of five medicinal plants of the Ayurvedic system on calcification in atherosclerosis [123] found that deposition of cholesterol and calcium on the elastic fiber, resulting in decreased elastin synthesis and cross-link formation, is directly related to calcification in smooth muscle cells hence gives rise to atherosclerosis. Calcium antagonists have been one of the most relevant therapeutic tools for patients with hypertension, and their effects on calcium transport may influence the cellular changes leading to atherosclerosis [124]. Calcification in arteriosclerosis is also inhibited by antioxidants. Thus, it was suggested that the BHUX-mediated reduction in the calcium content in
the atherosclerotic plaque could be attributed to the antioxidant property or to the calcium channel blocking property of *Terminalia arjuna* [125].

Apart from this, *Terminalia arjuna* is also well known for its high phenolic content. Thus, in the present study, bioactivity guided successive solvent extraction method was used to fractionate bioactive compounds from *Terminalia arjuna*. Here, bioactive compounds were separated on the basis of their polarity [93]. After performing froth test for the presence of saponins in butanol fraction, TLC showed the blue colored band confirming the presence of saponins. Saponin rich fractions of other plants like, *Herniaria hirsute* [126] has also been found to be a good inhibitor of calcium stone formation not only *in vitro* but *in vivo* too as found in present study. Saponin rich butanol fraction thus obtained after successive solvent extraction was administered to hyperoxaluric rats exposed to EG + $NH_4Cl$. Series of experiments done on urine and serum suggested preventive nature of saponin rich butanol fraction against urolithiasis. The results so obtained were very well supported by histopathological studies done with rat kidney tissue. Treated rats showed morphology like that of control group animals which were not given any treatment. In case of 9 days of treatment saponin rich butanol fraction showed 100% recovery when activity of alkaline phosphatase, a kidney injury marker enzyme, was measured. Main mode of action of inhibitors can be attributed to the structural fit between the biomolecule and the ionic structure of a particular crystal face. This decides the order of inhibition or reaction at a particular crystalline face. It may affect various crystalline faces differently depending upon the crystalline face exposed to the solution. As a result, it may change the morphology of the growing crystal. Small molecules with several negatively charged groups, such as citrate ions, do interact with the lateral faces of *CaP* and *CaOx* crystals. They slow down crystallization and invite changes in the crystal morphology.
Apart from this, there are various stone inhibitory proteins [127, 128] which are present in urine, having similar physical and chemical properties. Most of these proteins have been isolated from CaOx kidney stones matrix itself in their active form [129]. Likewise, many plants are also known to produce CaOx as crystalline deposits [130] having an organic matrix constituting different proteins. These proteins are believed to play an important role in the control of crystal growth and modification of crystal form. Thus, protein purification of Terminalia arjuna was further done. Again ultrafiltration was done and further purification was continued with greater than 10 kDa fraction. Enhanced inhibition was seen after each step of purification. Extent of purity was checked by running 10% SDS-PAGE of most potent fraction obtained after molecular sieve chromatography and an anionic protein having molecular weight ~ 14 kDa was isolated. Since, the proteins present in both Tamarindus indica and Terminalia arjuna inhibited CaP as well as CaOx crystal growth, a detailed study on the characterization of these proteins can impart a new direction to the treatment and cure of kidney stones.