ANTILITHIATIC EFFECT OF *TRIBULUS TERRESTRIS*: AN *IN VITRO* AND *IN VIVO* STUDY

Thesis submitted in fulfillment for the requirement of the degree of

Doctor of Philosophy

by

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DECLARATION

I hereby declare that the work reported in the Ph.D. thesis entitled **"Antilithiatic Effect of *Tribulus terrestris*: An *In Vitro* and *In Vivo* Study"** submitted at **Jaypee University of Information Technology, Waknaghat, India,** is an authentic record of my work carried out under the supervision of **Prof. C. Tandon**. I have not submitted this work elsewhere for any other degree or diploma. All assistance and help received during the course of the investigation has been duly acknowledged.

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CERTIFICATE

This is to certify that the work reported in the Ph.D. thesis entitled "**Antilithiatic Effect of *Tribulus terrestris*: An *In Vitro* and *In Vivo* Study**", submitted by **Anshu Aggarwal**, in the fulfillment for the award of degree of **Doctor of Philosophy** in **Biotechnology and Bioinformatics** by **Jaypee University of Information Technology, Waknaghat, India**, is a bonafide record of her original work carried out under our supervision. This work has not been submitted elsewhere for any other degree or diploma.

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To my parents

&

my sister "Rupali"

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Date:

Place: *Anshu Aggarwal*

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LIST OF ABBREVIATIONS

ALP	Alkaline phosphatase
ANOVA	Analysis of variance
B.C.	Before Christ
BCO	β-carotene 15, 15' monooxygenase
BLAST	Basic local alignment search tool
CaOx	Calcium Oxalate
CaP	Calcium Phosphate
CCD7	Carotenoid cleavage dioxygenase 7
cPLA$_2$	Cytosolic phospholipase A_2
CrCl	Creatinine clearance
DMEM	Dulbecco's modified Eagles's medium
DMSO	Dimethyl sulfoxide
EDTA	Ethylene diamine tetra acetic acid
EG	Ethylene Glycol
Egr-1	Early growth response protein 1
ESWL	Extracorporeal Shock Wave Lithotripsy
et al.	et alia (and others)
FBS	Fetal bovine serum
GAGs	Glycosaminoglycans
GFR	Glomerular Filtration Rate
HE	Hematoxylin/Eosin
HPLC	High Pressure Liquid Chromatography
i.p.	Intraperitoneally
IGD	In gel digest
JNK	c-Jun N-Terminal Kinase
LDH	Lactate dehydrogenase
LP	Low Pressure
MALDI-TOF	Matrix-Assisted Laser Desorption/Ionization –Time of Flight
MS	Mass Spectrometry
MAPK	Mitogen activated Protein Kinase
MDCK	Madin Darby Canine Kidney (Cell line)

NADH	Nicotinamide adenine dinucleotide
NCCS	National Centre of Cell Sciences
NH_4Cl	Ammonium Chloride
NRK-52E	Normal Rat Kidney Epithelial (Cell line)
PCNL	Percutaneous Nephrolithotomy
PMSF	Phenylmethanesulfonylfluoride
PTFE	Polytetrafluoroethylene
RBP4	Retinol Binding Protein
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
THP	Tamm-Horsfall Protein
TTP	*Tribulus terrestris* protein
URS	Ureterorenoscopy

ABSTRACT

Urolithiasis is the third most common disorder of the urinary tract affecting 2–20% population worldwide. Urinary calculi are caused by various etiological factors with a worldwide increasing incidence and prevalence causing substantial morbidity and economic burden. Archaeological findings give profound evidence that humans have suffered from kidney stones for centuries. Epidemiological data have shown that calcareous stones are found in a majority of kidney stones majorly composed of calcium oxalate and calcium phosphate. Calcium oxalate stones are found in two different varieties, calcium oxalate monohydrate or Whewellite, and calcium oxalate dihydrate or Weddellite. Calcium oxalate monohydrate, the thermodynamically most stable form, is observed more frequently in clinical stones than calcium oxalate dihydrate and it has a greater affinity for renal tubular cells, thus responsible for the formation of stones in kidney. The formation of such concretion encompasses several physiochemical events beginning with crystal nucleation, growth, aggregation and ending by retention within urinary tract. The mechanisms governing the induction of all these processes remain speculative. Surgical operation, lithotripsy and local calculus disruption using high-power laser are widely used to remove calculi along with dietary interventions and therapies like thiazide diuretics and alkali-citrate to prevent recurrence but scientific evidence for their efficacy is less convincing. Literature suggests that exposure to shock waves in therapeutic doses may cause acute renal injury, decrease in renal function and increase in stone recurrence. In addition, persistent residual stone fragments and the possibility of infection after ESWL represent a serious problem in the treatment of stones. Furthermore, in spite of substantial progress in the study of biological and physical manifestations of kidney stones, there is no satisfactory drug for use in clinical therapy. Therefore, it is

worthwhile to look for an alternative to these conventional methods by using phytotherapy. For thousands of years, man has sought healing powers from the natural world especially from plants. The recent resurgence of the plant remedies result from several factors like effectiveness of the plant based medicines and lesser side effects compared to modern medicine. Although information on the complete mechanism of action of each remedy is lacking, commonly known extracts exert antilithogenic properties by altering ionic composition of urine, by expressing diuretic activity, having antioxidant activity or anti-inflammatory role. The plants are also observed to exert antilithogenic potency due to the interplay of certain biomolecules in restoration of renal functioning. There are few composite herbal drugs *viz.* Cystone, Neeri, Uriflow which are used to treat urolithiasis.

Tribulus terrestris, commonly called as "gokhru", is a widely distributed plant worldwide and is used as a traditional medicine for treatment of urolithiasis. It is also an active component of marketed antiurolithiatic drugs like cystone, neeri, uriflow. The present study is aimed at evaluating the inhibitory potency of *Tribulus terrestris* on calcium oxalate crystallization *in vitro* and *in vivo* along with the isolation and characterization of biologically active compounds from *Tribulus terrestris* to establish its scientific basis.

The activity of aqueous extract of *Tribulus terrestris* was investigated on nucleation and growth of the calcium oxalate (CaOx) crystals as well as on oxalate induced cell injury of renal epithelial cells (NRK-52E). *Tribulus terrestris* extract exhibited a concentration dependent inhibition of nucleation and the growth of CaOx crystals. When NRK-52E cells were injured by exposure to oxalate for 72 h, *Tribulus terrestris* extract prevented the injury in a dose-dependent manner. On treatment with the

different concentrations of the plant, the cell viability increased and the LDH release decreased in a concentration dependent manner. The current data suggests that *Tribulus terrestris* extract not only has a potential to inhibit nucleation and the growth of the CaOx crystals but also has a cytoprotective role.

The efficacy of the aqueous extract of *T. terrestris* was validated using a rat urolithiatic model in prophylactic and curative regimen. Hyperoxaluria was induced in rats using ethylene glycol (0.4%) and ammonium chloride (1%) for 15 days and was then replaced by only EG (0.4%) in curative regimen for another 13 days post-prophylactic regimen. Lithogenic treatment impaired renal function as evident from increase in serum urea, serum creatinine and decrease in creatinine clearance. Exposure to EG and NH_4Cl also led to significant damage to renal tubules as there was a marked increase in the activity of renal injury markers (Alkaline phosphatase and Lactate dehydrogenase) in urine. Upon administration of aqueous extract of *T. terrestris* (50 mg/kg body wt and 100 mg/kg body wt), levels of renal injury markers were normalized with a decrease in serum urea and serum creatinine and an increased creatinine clearance. The histopathological analysis supported the protective effect of *T. terrestris* as its administration was able to reduce the dilatation of proximal tubules, glomerular damage along with a decrease in the irregular crystals present in the tubules. Crystalluria analysis showed that there was a decrease in size of the crystals, emphasizing that the plant facilitates expulsion of crystals by breaking them into smaller crystals in prophylactic regimen; however, in curative regimen plant extract administration led to decrease in number of crystals thus decreasing supersaturation and sites for aggregation.

Using conventional bio-activity guided purification technique involving ammonium sulphate precipitation, anion exchange chromatography and molecular sieve chromatography combined with mass spectrometry, a novel antilithiatic protein was purified from the fruits of *Tribulus terrestris*. The protective potency of the protein was tested on the oxalate induced injury on renal epithelial cell lines (NRK-52E). An antilithiatic protein (TTP) having molecular weight of ~ 60kDa was purified. This purified protein which on amino acid analysis was found to be tyrosine rich, showed similarities with Carotenoid cleavage dioxygenase 7 (CCD7) of *Arabidopsis thaliana* after matching peptide mass fingerprints in MASCOT search engine. CCD7 belong to the family of proteins involved in the formation of retinol using tyrosine in their catalytic site, deficiency of which lead to calculus formation. Presence of an EF hand domain, a characteristic feature of calcium binding proteins in CCD7 further corroborates the role of TTP as an antilithiatic protein. The protective potency of 4µg/ml TTP on NRK-52E was quite comparable to the 50µg/ml aqueous extract of *T. terrestris*.

The studies with aqueous extract of *Tribulus terrestris* indicate that it has the potential of inhibiting calcium oxalate crystallization, influencing crystal-cell interaction and preventing and curing kidney stones. The studies also suggest that the purified protein from *Tribulus terrestris* is endowed with an antilithiatic potential.

INTRODUCTION

Biomineralization, which refers to the complex processes by which organisms form minerals, is frequently associated with a high degree of regulation on different hierarchical levels. "Calcium stone formation" is mineral crystallization in body tissue or fluid. The deposition of inorganic minerals, crystalline or noncrystalline, around biomolecules is universal in biology, where inorganic crystals are harnessed to become an integral part of organic tissue to provide hardness and strength. More than 200 yr ago, Hunter articulated about the similarity between stone formation and calcification pointing the equivalence of enamel, eggshell, gallstones, and kidney stones (Hunter, 1771). Thus, mineralization can be arbitrarily divided into physiologic and pathologic. Physiologic crystallization includes formation of exoskeleton, pearl, endoskeleton, and dentition, whereas pathophysiologic crystallization includes pyrophosphate arthropathy, pigmented gallstones, vascular calcification, and urolithiasis. Pathologic calcium crystallization is a physiologic process in the wrong place and the wrong time (Moe and Bonny, 2005). The majority of normal and pathological bio-minerals formed in humans are sparingly soluble electrolytes with basic anions (i.e. anions that can be protonated), such as phosphates, carbonates, oxalates, urates, etc. Their solubility thus, depends (often strongly) on pH (Königsberger and Königsberger, 2006).

Urolithiasis, i.e. the formation of stones or calculi in the urinary tract, is not only a painful condition affecting some 2 – 20% of the population worldwide but 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). The term *urolith* is derived from the Greek *ouron* meaning urine, and *lithos* meaning stone (Osborne *et al.*, 1999). The oldest urolith of human origin was found in the Egyptian tomb of a 16-year-old

boy and dates from around 4800 B.C. (Ellis, 1979). Reference to stone formation is made in the early Sanskrit documents in India between 3000 and 2000 B.C. (Prasad *et al.*, 2007). As it is clear from these historical clues, urinary stone has always been a common disease and presently it is the third most common affliction of the urinary tract (Atmani, 2003). Depending on the socio-economic conditions and subsequent changes in the dietary habits, the overall probability of stone formers differs in various part of world: 1-5% Asia, 5-9% Europe, 13-15% USA and 20% Saudi Arabia. The "stone belts" of the world are located in the countries of the Middle East, North Africa, Mediterranean regions, North-western states of India and Southern states of USA (Lopez and Hoppe, 2010). In India, with a prevalence rate of 15%, two high incidence stone belts have been found to occur. The first belt starts from Amritsar in North and while passing through Delhi and Agra ends up in U.P. The other belt which starts from Jamnagar in west coast extends inwards towards Jabalpur in central India. Very low incidence areas have been in West Bengal and coastal areas of Maharashtra, Karnataka, Kerala, Tamil Nadu, Andhra Pradesh (Rizvi *et al.*, 2002; Tandon *et al.*, 1999).The incidence of urinary stones has been increasing over the last few years while the age of onset is decreasing (Devuyst and Pirson, 2007). With the prevalence rate of >10% and an expected recurrence rate of ~50%, stone disease has an important effect on health care system (Knoll, 2007). For decades, urolithiasis has arguably been one of the most research intensive sectors of clinical and fundamental investigations into the cause, prevention and treatment of crystal deposition diseases in humans. However, it appears that a real breakthrough in this area is lacking as yet.

The formation of uroliths involves multiple physiological and pathological processes. Human body fluids are normally supersaturated with regard to several substances (e.g.

blood plasma, interstitial and intracellular liquors with respect to calcium carbonates and phosphates, particularly hydroxyapatite and fluoroapatite; bile with respect to cholesterol; urine with respect to calcium oxalates and, depending on the pH, with regard to uric acid or calcium phosphates). The question, why crystalluria is common but stone formation is not, has been discussed in terms of three main factors:

(i) the supersaturation as a necessary condition,

(ii) the presence of heterogeneous nucleants and

(iii) deficit of crystallization inhibitors (Grases and Costa-Bauza, 1999)

In a comprehensive review, different types of renal stones have been classified (Grases *et al.*, 1998). These stones or calculi are majorly composed of calcium oxalate hydrates (calcium oxalate monohydrate and calcium oxalate dihydrate), ammonium magnesium phosphate (struvite), calcium phosphates (hydroxyapatite and brushite), uric acid and urates, cystine and xanthine. Figure 1.1 depicts various types of stones majorly found in human population alone or in combination.

The etiology of this disorder is multifactorial and is strongly related to dietary, lifestyle habits and climatic changes (Lopez and Hoppe, 2010). Management of stone disease depends on the size of calculi, severity of symptoms, degree of obstruction, kidney function, location of the stones and the presence or absence of associated infection influence the choice of one type of intervention over the other and ranges from observation (watchful waiting) to surgical removal of the stone.

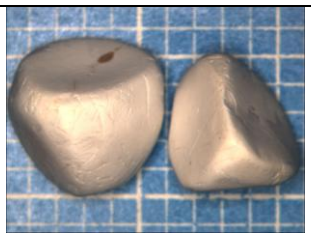
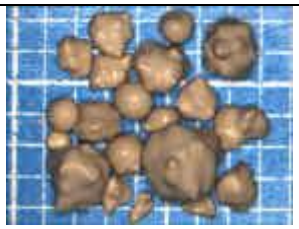
 Calcium oxalate monohydrate		 Calcium oxalate dihydrate
 Struvite		 Hydroxyapatite
 Brushite		 Uric acid
 Ammonium Acid Urate		 Cystine
 Xanthine		 2,8-dihydroxyadenine

Figure 1.1: Kidney Stones

Stones larger than 5mm fail to pass through and require assistance medically (Butterweck and Khan, 2009). Currently this serious problem can be treated with increased fluid intake, medications like thiazides, allopurinol (Atmani, 2003) and potassium citrate (Tracy and Pearle, 2009) for reducing urinary saturation of stone forming calcium salts and thus preventing nucleation, crystallization and agglomeration of calcium salts, diuretics for facilitating urine flow, surgical 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).

Despite major technical advancements in treating kidney stones, the problem still persists. Treatments available till date have serious side effects and do not eliminate the probability of recurrence completely. 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 effects as renal damage and hypertension (Butterweck and Khan, 2009). Furthermore, ESWL is known to be related to long term medical effects such as diabetes mellitus (Krambeck *et al.*, 2006). Even the medications like thiazides cause intracellular acidosis and can lead to hypokalemia and hypocitraturia (Heilberg and Schor, 2006).

Actually, there are no satisfactory drugs in modern medicine, which can dissolve stone and the physicians remain to be dependent on alternative systems of medicine for better relief. Herbal medicines are efficacious and have lesser side effects compared to modern medicines and also reduce the recurrence rate of renal stone (Prasad *et al.*, 2007). The complete mechanism of action of these remedies is lacking.

Unlike allopathic medicines which majorly target only one aspect of urolithiatic pathophysiology, most of the plant based therapies have been shown to be effective at different stages of stone pathophysiology. Currently known extracts exert their antilithogenic properties by multiple mechanisms like:

- Help in spontaneous passage of calculi by increasing urine volume, pH and anti-calcifying activity (Diuretic activity)
- Balance the inhibitor and promoters of crystallization in urine and affects the crystal nucleation, aggregation and growth (Crystallization inhibition activity)
- Relieve the binding mucin of calculi (Lithotriptic activity)
- Improve renal function
- Regulate oxalate metabolism
- Regulate the crystalloid colloid imbalance and improve renal function, thus prevents recurrence of urinary calculi
- Improve renal tissue antioxidant status and cell membrane integrity and prevents recurrence (Antioxidant activity)
- Exert significant anti-infective action against the major causative organisms (Antimicrobial activity)
- Relieve pain, burning micturition and haematuria (Analgesic and anti-inflammatory activity) (Pareta *et al.*, 2011)

The marketed composite herbal formulations, Cystone (Himalaya Drug Company, India), Neeri (Aimil Pharmaceuticals, India), Uritone (Aimil Pharmaceuticals, India), Uriflow (Bioneutrix Labs), Culdisol (Ganga Pharmaceuticals, India), Calcury (Charak Pharmaceuticals, India), Chandraprabhabati (Baidyanath, India) and Culin Forte (Alopa Herbal) have been used worldwide to dissolve urinary calculi in kidney and

urinary bladder. *Tribulus terrestris* has long been an important constituent in tonics in Indian Ayurveda practice, where it is known by its Sanskrit name, "gokshura". This is a common constituent of antiurolithiatic herbal formulations like Cystone, Neeri, Uritone, Uriflow, Culdisol and Culin forte.

Tribulus terrestris (Puncture Vine, Caltrop, Yellow Vine and Gokhru) is a flowering plant of the Zygophyllaceae family, native to warm temperature and tropical regions of the old world in Southern Europe, Southern Asia, Africa and Northern Australia. It can thrive even in desert climates and poor soil.

Figure 1.2: *Tribulus terrestris*

The roots and fruits are sweet, cooling, diuretic, aphrodisiac, emollient, appetiser, digestive, anthelmintic, expectorant, anodyne, anti-inflammatory, alterant, laxative, cardiotonic, styptic, lithotriptic and tonic. They are useful in strangury, dysuria, vitiated conditions of vata and pitta, renal and vesical calculi, anorexia, dyspepsia, helminthiasis, spermatorrhoea, anaemina, scabies, ophthalmia and general weakness. The leaves are astringent, diuretic, aphrodisiac, depurative, anthelmentic and tonic. They are useful in gonorrhoea, inflammation, menorrhagia, strangury, leprosy, skin

diseases, verminosis and general weakness. The seeds are astringent, strengthening and are useful in epistaxis, haemorrhages and ulcerative stomatitis. The ash of the whole plant is good for external application in rheumatoid-arthritis (Warrier *et al.*, 1996). The diuretic properties of the plant are due to the large quantities of the nitrates present as well as the essential oil which occurs in the seeds (Nadkarni, 1993).

In a preliminary study, the diuretic effect of *Tribulus terrestris* and *Hygrophila spinosa* water extracts in albino rats was evaluated (Kumari and Iyer, 1967; Singh *et al.*, 1991). The effect of an aqueous extract of *Tribulus terrestris* administered orally at a dose of 5 g/kg body wt and restoration in urinary oxalate was observed (Sangeeta *et al.*, 1993). Few studies were conducted to evaluate the therapeutic use of *Tribulus terrestris* in various urinary disorders including urolithiasis (Anand *et al.*, 1994; Sangeeta *et al.*, 1994).The inhibitory potency of the extract of putatively litholytic plant, *Tribulus terrestris* was tested on the growth of brushite and CaOx crystals *in vitro* and it exhibited appreciable amount of inhibition (Joshi *et al.*, 2005a; 2005b).

As discussed above, various marketed antiurolithiatic herbal formulations like Cystone (Himalaya Drug Company), Neeri (Aimil Pharmaceuticals), Uritone (Aimil Pharmaceuticals), Uriflow (Bioneutrix), Culdisol (Ganga Pharmaceuticals) and Culin forte (Alopa Herbal) contain *Tribulus terrestris* as a common constituent in different proportions.

Keeping in view the importance of *Tribulus terrestris* as an antiurolithiatic agent and the complications arising due to the surgical treatment of kidney stones available, the study was designed to further investigate its antilithiatic potency *in vitro* and *in vivo* with the following objectives:

1. To study the effect of aqueous extract of *Tribulus terrestris* on calcium oxalate crystallization.
2. To study the effect of *Tribulus terrestris* on oxalate induced injury in rat renal epithelial cell lines (NRK-52E).
3. To investigate the prophylactic and curative role of aqueous extract of the *Tribulus terrestris* on experimentally induced nephrolithiatic rats.
4. To isolate, purify and characterize the new biologically active compounds (potent biomolecules) from *Tribulus terrestris* which have the ability to influence calcium oxalate crystal growth.

REVIEW

OF

LITERATURE

Urolithiasis has afflicted mankind for millennia and continues to be a significant medical ailment throughout the world. Contemporary management reflects the changes and evolution that have occurred in urology. Traditional open surgery has been nearly replaced by minimally invasive techniques which are the result of technologic innovations, miniaturization of instruments, and interdisciplinary collaboration. Nevertheless, nephrolithiasis remains a chronic disease and our fundamental understanding of the pathogenesis of stones as well as their prevention and cure, remains rudimentary.

2.1 Urolithiasis

2.1.1 Overview

Urolithiasis, referred to the formation of stone in the urinary tract (Figure 2.1), is one among diseases that afflicted humans since antiquity. Despite tremendous advances accomplished in understanding the mechanisms governing the formation of such concretion, the disease remains an enigma since several factors intervene and interfere with each other. Kidneys reabsorb water and contribute to the concentration of different solutes that might crystallize and precipitate. As many forms of mineralization, urolithogenesis encompasses several physico-chemical steps which occur either sequentially or concurrently starting with supersaturation, then nucleation, growth and aggregation. Of course, based on their size, aggregated particles can be attached and retained within urinary tubule epithelium constituting an additional step in calculogenesis (Daudon *et al.*, 1995; Khan, 1997). Urolithiasis is increasing in prevalence and causes substantial morbidity and economic burden (Sayer *et al.*, 2010). Urolithiasis is a global problem spanning all geographic regions with an estimated annual incidence of 1%, prevalence of 3–5% and a lifetime risk of

15–25%. Once afflicted, urolithiasis tends to be recurrent in the majority of cases. According to data from the Urological Diseases in America Project, the total annual cost of nephrolithiasis in the United States in the year 2000 was about $5.3 billion. This underscores the toll taken by this disease on working-age individuals and society at large (Moe *et al.*, 2011). According to the clinical and epidemiological studies, calcium oxalate (CaOx) followed by calcium phosphate (CaP) are the most frequently encountered crystalline components found in the analyzed stones (Daudon *et al.*, 1995). Most stones do not contain one single crystal phase but rather a mixture of several different crystal phases. Given the high cost of urgent medical treatment and/or surgical intervention, the attractiveness of a medical prophylactic program to reduce stone occurrences or increase the likelihood of successful conservative management of an acute-stone event is obvious.

2.1.2 Kidney Function

The urinary system is the organ system that produces, stores, and eliminates urine. In humans it includes two kidneys, two ureters, the bladder, and the urethra (Figure 2.2) (O'Callaghan, 2006). The kidneys lie behind the peritoneum at the back of the abdominal cavity. The kidney performs the essential function of removing waste products and maintaining body homeostasis. The renal cortex is the outer zone of the kidney and the renal medulla is the inner zone which is made up of the renal pyramids. The cortex contains all of the glomeruli and the medulla contains the loops of Henle, the vasa recta and the final portions of the collecting ducts. The nephron is the basic unit of the kidney and each kidney has 400,000-800,000 nephrons, although this number declines with age (O'Callaghan, 2006; Delvecchio and Preminger, 2003). A nephron consists of a glomerulus and an associated tubule that leads to the

collecting duct. The urinary filtrate is formed in the glomerulus and passes into the tubules where the volume and content are altered by reabsorption or secretion. Most solute reabsorption occurs in the proximal tubules; whereas fine adjustments to urine composition are later made in the distal tubule and collecting ducts. The loop of Henle serves to concentrate urine. In the proximal tubules, glucose, sodium chloride, and water are reabsorbed and returned to the blood stream, along with essential nutrients such as amino acids, proteins, bicarbonate, calcium, phosphate, and potassium. In the loop of Henle, the urine concentrating process proceeds and in the distal tubule the salt- and acid-base balance of blood is regulated. The final urine is formed in the collecting ducts, where the urine is drained into the calyces that lead to the ureter (O'Callaghan, 2006).

2.1.3 History

Kidney and urinary tract stones have tormented humans for ages. The history of urolithiasis dates back to the dawn of civilization. The oldest urolith of human origin was found in the Egyptian tomb of a 16-year-old boy and dates from around 4800 B.C. (Ellis, 1979). Reference to stone formation is made in the early Sanskrit documents in India between 3000 and 2000 B.C. (Prasad *et al.*, 2007). The symptoms of this disease are reported by many famous historical figures, for this condition affected all groups of society. Famous people who suffered from urolithiasis include King Leopold I of Belgium, Napoleon Bonaparte, Emperor Napoleon III, Peter the Great, Louis XIV, George IV, Oliver Cromwell, Benjamin Franklin, the philosopher Bacon, the scientist Newton, the physicians Harvey and the botanist Boerhaave and the anatomist Scarpa (Basler *et al.*, 2011).

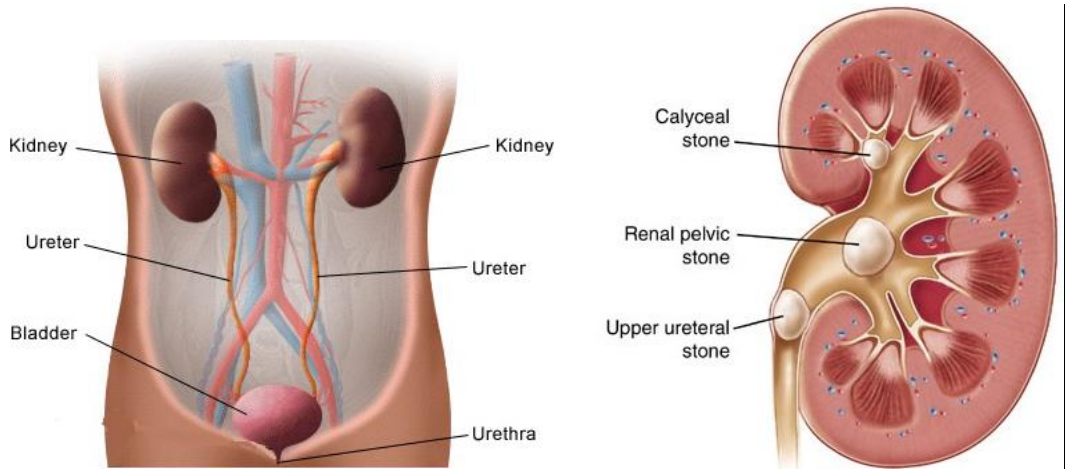


Figure 2.1: Common location of stones in Urolithiasis

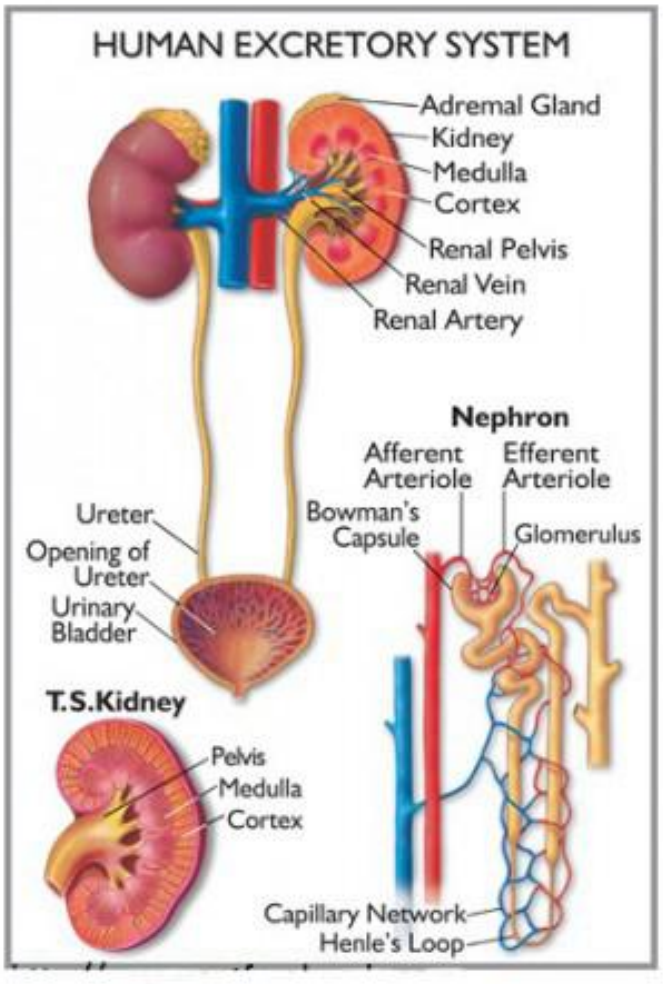


Figure 2.2: Human Excretory System

2.1.4 Epidemiology

As it is clear from these historical clues, urinary stone has always been a common disease and presently it is the third most common affliction of the urinary tract (Atmani, 2003) with a worldwide prevalence of between 2 – 20% (Johri *et al.*, 2010). Depending on the socio-economic conditions and subsequent changes in the dietary habits, the overall probability of stone formers differs in various part of world: 1-5% Asia, 5-9% Europe, 13-15% USA and 20% Saudi Arabia. The "stone belts" of the world are located in the countries of the Middle East, North Africa, Mediterranean regions, North-western state of India and Southern state of USA (Lopez and Hoppe, 2010). In India, with a prevalence rate of 15% (Rizvi *et al.*, 2002), two high incidence stone belts have been found to occur. The first belt starts from Amritsar in North and while passing through Delhi and Agra ends up in U.P. The other belt which starts from Jamnagar in west coast extends inwards towards Jabalpur in central India. Very low incidence areas have been in West Bengal and coastal areas of Maharashtra, Karnataka, Kerala, Tamil Nadu, Andhra Pradesh (Tandon *et al.*, 1999).The incidence of urinary stones has been increasing over the last few years while the age of onset is decreasing (Devuyst and Pirson, 2007). With the prevalence rate of >10% and an expected recurrence rate of ~50%, stone disease has an important effect on health care system (Knoll, 2007).

2.2 Pathophysiology of Urolithiasis

Renal stone disease is not a single disorder since stone composition varies, which reflects constitutional, environmental and genetic factors. Renal calculi are crystalline mineral deposits that form in the kidney. They develop from microscopic crystals in the loop of Henle, the distal tubule, or the collecting duct and they can enlarge to form

visible fragments. The process of stone formation depends on urinary volume; concentrations of calcium, phosphate, oxalate, sodium, and uric acid ions; concentrations of natural calculus inhibitors (e.g., citrate, magnesium, Tamm-Horsfall mucoproteins, bikunin); urinary pH; retention of crystals in the kidney (Mandel, 1996; Verkoelen *et al.,* 1997).

Table 2.1 lists the main types of renal stones and their relative prevalence (Prasad *et al.*, 2007).

Type of stones	Percentage prevalence
Calcareous stones (Calcium oxalate stones and Calcium phosphate stones)	75-90%
Magnesium Ammonium Phosphate (Struvite stones)	10-15%
Uric acid stones	3-10%
Cystine and other stones	0.5-1%

Table 2.1: Main types of renal stones and their relative prevalence

2.2.1 Calcium oxalate stones (monohydrate or dihydrate)

Calcium oxalate stones are the most common type of urinary calculi and can exist in monohydrate and dihydrate forms (Figure 2.3, Figure 2.4), with or without phosphate. High phosphate content may be associated with higher recurrence rates. Calcium oxalate stones are radiopaque and usually visible on plain film radiography or noncontrast CT. Hypercalciuria (i.e., more than 250 mg per 24 hours [6.2 mmol per day]) (Menon and Koul, 1992) is the most common metabolic abnormality associated with these calculi. Other causes of calcium oxalate stones include hyperoxaluria (i.e., more than 45 mg per 24 hours [500 μmol per day]), hypocitraturia (i.e., less than 450 mg per 24 hours [2.34 mmol per day]), which involves a deficiency of the naturally

occurring stone inhibitor citrate, and hyperuricosuria (i.e., more than 800 mg per 24 hours [4.76 mmol per day]) (Pietrow and Karellas, 2006). Hypercalciuria is heterogeneous in origin and three types existed: 1) absorptive hypercalciuria, in which the primary abnormality is an increased intestinal absorption of calcium; 2) renal hypercalciuria, characterized by a primary renal wasting of calcium; and 3) resorptive hypercalciuria, characterized by increased bone demineralization (Menon and Koul, 1992). Oxalate is an unavoidable component of the human diet as it is a ubiquitous component of plants and plant-derived foods. Endogenous oxalate synthesis (Figure 2.5) primarily occurs in the liver with glyoxylate as an immediate oxalate precursor. Glyoxylate is derived from oxidation of glycolate by glycolate oxidase or by catabolism of hydroxyproline, a component of collagen. Transamination of glyoxylate with alanine, by alanine/glyoxylate aminotransferase (AGT), results in the formation of pyruvate and glycine. Excess glyoxylate, however, will be converted to oxalate by glycolate oxidase or lactate dehydrogenase, of which the latter most likely catalyzes the bulk of this reaction. Hyperoxaluria can be generally divided into two categories: primary and secondary hyperoxaluria. Primary hyperoxaluria is the result of inherited (mostly) hepatic enzyme deficiencies leading to increased endogenous oxalate synthesis. Secondary hyperoxaluria results from conditions underlying increased intestinal oxalate absorption, such as (1) a high-oxalate diet, (2) fat malabsorption (enteric hyperoxaluria), (3) alterations in intestinal oxalate degrading microorganisms, *Oxalobacter formigenes* and (4) genetic variations of intestinal oxalate transporters (Robijn *et al.*, 2011). Another source of oxalate is the catabolism of ascorbate (vitamin C) in the urine or blood. Ascorbate can be oxidized by a variety of enzymatic and nonenzymatic pathways to dehydroascorbate, which then breaks down

nonenzymatically to L-erythrulose or L-threonate, carbon dioxide, and oxalate (Linster and Van Schaftingen, 2007). Although some reports suggest that ascorbate increases oxalate excretion (Massey *et al.,* 2005), other work indicates that ascorbate decreases the risk of nephrolithiasis overall by binding to calcium and thereby reducing urinary calcium oxalate supersaturation (Micali *et al.,* 2006). The cause of hypocitraturia often is idiopathic, although high dietary acid loads (e.g., from excessive meat intake) and dehydration can exacerbate this condition (Pietrow and Karellas, 2006). Hyperuricosuria, being a risk factor for calcium oxalate stone disease can be attributed to several factors, including promotion of heterogeneous nucleation by uric acid or monosodium urate, blocking of some of the inhibitory effects of urine macromolecules on calcium oxalate crystal formation, or a lowering of the limit of metastability for calcium oxalate by uric acid (Grover *et al.*, 2003).

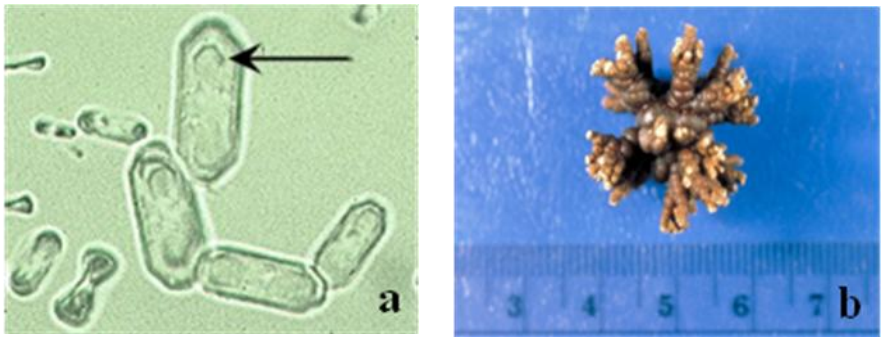


Figure 2.3(a): Microscopic view of calcium oxalate monohydrate (Whewellite). Figure 2.3(b): Macroscopic view of calcium oxalate monohydrate (Whewellite)

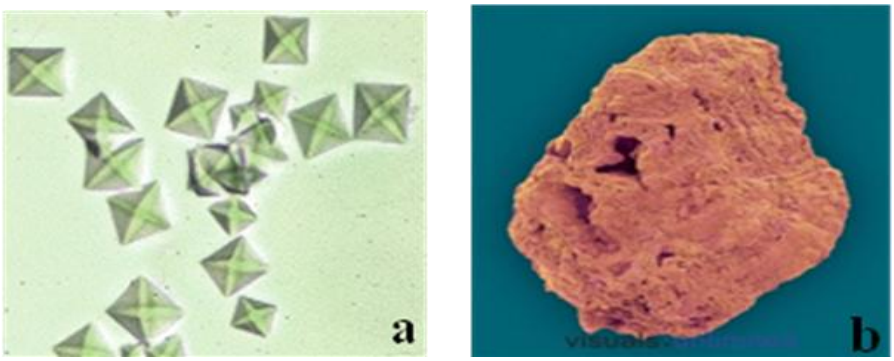


Figure 2.4(a): Microscopic view of calcium oxalate dihydrate (Weddellite). Figure 2.4(b): Macroscopic view of calcium oxalate dihydrate (Weddellite)

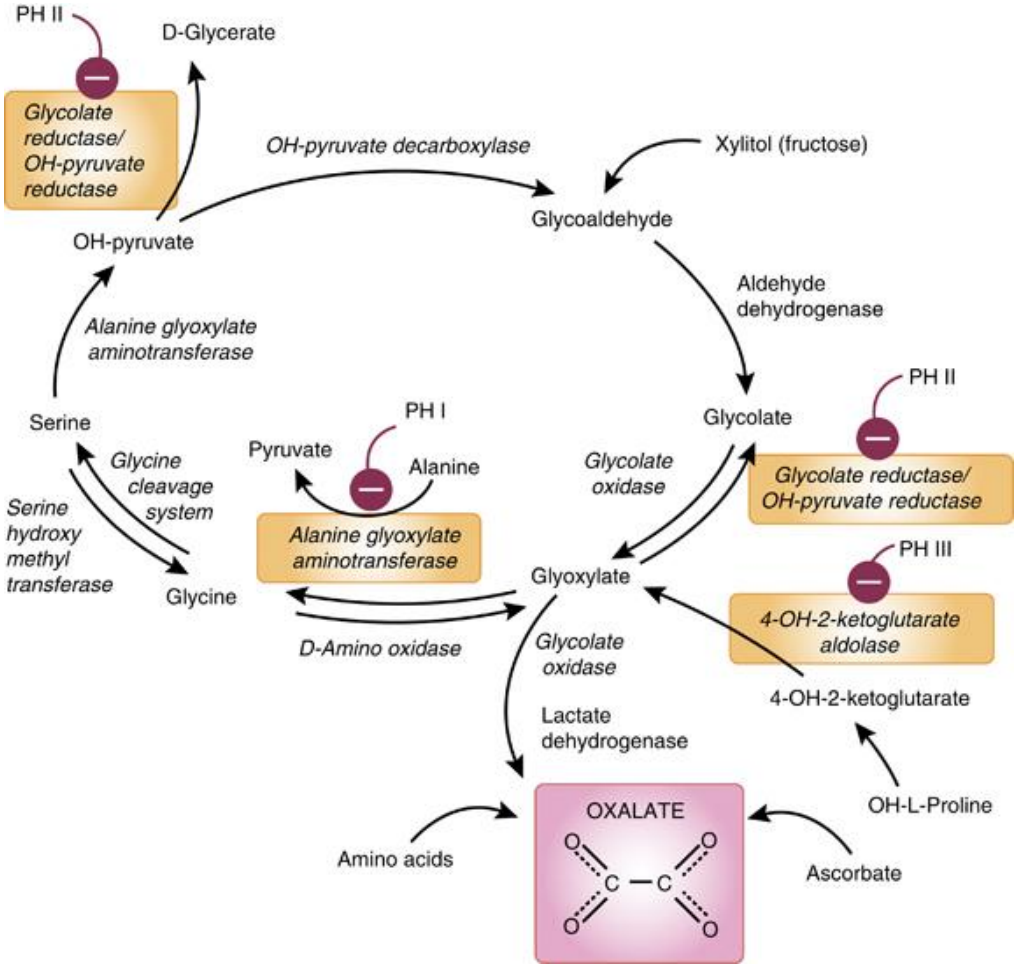


Figure 2.5: Overview of endogenous oxalate synthesis pathways. PH I–III, primary hyperoxaluria types I–III.

2.2.2 Calcium phosphate stones

Calculi that consist predominantly of calcium phosphate occur more often in women than in men. Reports indicate a higher rate of recurrence in stones with a greater fraction of calcium phosphate (Tiselius, 1992). They often are associated with acidification disorders such as renal tubular acidosis which is associated with hypercalciuria and hypocitraturia. Medical treatment of these stones consists of replenishing urinary citrate to prevent new stone formation and delay growth of existing stones (Pietrow and Karellas, 2006). Patients with stone disease often manifest high levels of urinary phosphate excretion, either as a result of excessive dietary intake or due to a renal phosphate leak as a component of the syndrome of absorptive hypercalciuria (Williams *et al.*, 1996).

2.2.3 Struvite stones

Struvite stones, also known as infection or triple-phosphate stones consist of magnesium, ammonium, and calcium phosphate. They occur more often in women than in men and are the leading cause of staghorn calculi. Neurogenic bladders and foreign bodies in the urinary tract also predispose patients to struvite calculi. Recurrent urinary tract infections with urea-splitting organisms (e.g., *Proteus mirabilis, Ureaplasma urealyticum, Klebsiella pneumoniae*) result in alkalinization of urine and the addition of ammonium to the milieu. The prerequisites are thereby fulfilled for the precipitation of both magnesium ammonium phosphate and carbonate apatite. The formation product of magnesium ammonium phosphate is $2.5 \times 10^{-13}(mol/L)^3$. Struvite stones are usually radiopaque on standard radiographic imaging but may be quite faint (Tiselius, 2003; Pietrow and Karellas, 2006).

2.2.4 Uric acid stones

Uric acid stones may consist of uric acid only, or they also may contain calcium (Moe *et al.*, 2002). Uric acid is a by-product of ingested or endogenous purine metabolism and is excreted in the urine primarily in insoluble form. The primary cause of uric acid stones is a urinary pH below the p*Ka* for uric acid (5.5) along with high excretion of urate and small urine volume. Uric acid can precipitate at normal urate concentrations if the pH is sufficiently low; the formation product of uric acid is 5.0 X $10^{-9}(mol/L)^2$ (Tiselius, 2003). Men with gout have a twofold risk of having a uric acid calculus (Kramer *et al.*, 2003). Excess ingestion of animal meat protein (i.e., meat of all types, including poultry) can be detected by measuring urinary sulfate levels. Radiographic imaging can be difficult because pure uric acid calculi typically are radiolucent. They are, however, readily apparent on non-contrast CT.

2.2.5 Cystine stones

Patients with cystine calculi have an autosomal recessive disorder of dibasic amino acid transport leading to decreased cystine resorption in the kidney. Only homozygote patients form cystine calculi and often present with stones during childhood. Calculi may be pure cystine or may be mixed with calcium oxalate. Cystine is poorly soluble at normal urinary pH and will readily form stones when levels rise above a concentration of 250 mg/L or at an ion-activity product of >1.3 X 10^{-20} $(mol/L)^3$. Pure cystine stones are yellow and radiolucent (Tiselius, 2003; Pietrow and Karellas, 2006).

2.3 Etiology of Urolithiasis

2.3.1 Risk Factors

The risk factors involved in kidney stone formation might influence the clinical course of the disease. Risk factors are generally divided into non-dietary (Family History, Age and Sex, Environmental factors), dietary and urinary factors.

2.3.1.1 Non-Dietary Factors

Studies of twins and populations have demonstrated that the common forms of stone disease are heritable (Goldfarb *et al.*, 2005). Their study done on dizygotic and monozygotic twins showed that the heritability of the risk for the stones was 56%. The risk of stone formation is twofold higher in individuals with a family history of stone disease (Curhan *et al.*, 1997). The increased risk is likely due to both genetic predisposition and similar environmental exposures (e.g., diet). Genetic causes of rare forms of nephrolithiasis (e.g., cystinuria, Dent disease) have been identified, but information is still limited on genes that contribute to risk of the common forms of stone disease (Skopkova *et al.*, 2005; Devuyst, 2004). A slightly higher rate of renal stone disease emerged in males than in females, and in white Caucasians than in Blacks. Stones in the upper urinary tract appear to be related to the life-style, being more frequent among affluent people, living in developed countries, with high animal protein consumption. Bladder stones are nowadays mainly seen in the Third World, on account of very poor socio-economic conditions. A high frequency of stone formation among hypertensive patients has been reported, and among those with high body mass as well (Ramello *et al.*, 2000). Previous studies suggest a familial incidence in a subset of persons who have recurrent urinary tract stone disease. Identification and characterization of families of recurrent stone formers is essential

for the identification of unique genetic, environmental and metabolic factors that predispose individuals to recurrent CaOx stone formation. As oxaluria and calciuria have a prominent role in calcium stone formation, any genes that influence their excretion can be considered prime candidates in calcium nephrolithiasis. Genetic studies have identified a small group of individuals with known metabolic disorders who develop recurrent CaOx stones at a very early age. Recently, a new suggestive gene locus for autosomal dominant nephrolithiasis, have been discovered. It is localized on chromosome 9q33.2-q34.2. The responsible gene will provide new insight into the molecular basis of nephrolithiasis (Wolf *et al.*, 2005). Low serum phosphate concentrations due to decrease in renal phosphate reabsorption have been reported in some urolithiatic patients with defect in gene coding for the type 2a sodium-phosphate co-transporter (Prie *et al.*, 2002).

The inhibition of CaOx crystal growth is influenced by a complex combination of gender and age. With age, the ability to inhibit crystallization is reduced. Men are at higher risk for kidney stones than women. The daily excretion of magnesium and zinc is higher in men due to higher body weight of men. The citrate:creatinine excretion ratio was higher in women explaining lower incidence of stones in women (Trinchieri *et al.*, 1992). Another reason for lower occurrence of stones in women is alpha-trypsin timer which is CaOx crystallization inhibitor and a function of age and sex hormone status in males and females. With increase in age, males show a decrease in alpha trypsin timer but females don't show this decrease (Ricchiuti *et al.*, 2002).

While determining the geographic variability in the rates of the kidney stones in the Unites States, it was found that ambient temperature and sunlight levels are important risk factors for stones. The differences in exposure to temperature and sunlight may

contribute to geographic variability. The reasons for higher incidence in summers could be due to an increased conversion of vitamin D3 to its active metabolites resulting in increased calcium absorption from intestines and decrease in urine production due to loss of water as sweat, causing supersaturation of urine with stone constituents (Soucie *et al.*, 1996). 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).

2.3.1.2 Dietary Factors

Diet-related factors that are known to increase stone risk are listed in Table 2.2. Tea or coffee (particularly instant coffee) without milk has been shown to increase oxalate excretion, although this effect is probably offset by their diuretic action. Dietary calcium has a biphasic risk curve: stone risk is greater in those on a high or low calcium diet. Vitamin C excess could also increase the risk of calcium oxalate stone formation, but in practice this is rarely encountered. High dietary intake of potassium or magnesium is inversely related to stone formation because potassium promotes urinary citrate excretion, and both citrate and magnesium inhibit crystal formation (Taylor *et al.*, 2004; Johri *et al.*, 2010). It was also suggested that idiopathic renal stone genesis could be generated by Vitamin A deficiency (Sakly *et al.*, 2003).

Dietary Risk Factors
Low fluid intake
High intake of animal protein
High dietary sodium
Excessive intake of refined sugars
Foods rich in oxalate
High intake of grapefruit juice, apple juice and soft cola drinks

Table 2.2: Dietary risk factors associated with increased stone risk

2.3.1.3 Urinary factors

Urinary factors include condition and composition of urine which could result in formation of kidney stones. These factors can be concentration of ions in urine, volume of urine, pH of urine, enzymes concentration and level of various stone formation inhibitors in urine.

The key process in the development of kidney stones is supersaturation. This process involves salts that are carried in urine such as calcium oxalate, uric acid, cystine or xanthine. If the volume of urine is significantly reduced or if abnormally high amounts of crystal-forming salts are present, they precipitate out and form crystals. An *in vitro* study has shown that oxalate, either in crystalline or in soluble form, triggers a spectrum of responses in renal cells that favor stone formation, including alteration in membrane surface properties that promote crystal attachment and alterations in cell viability that provide debris for crystal nucleation. The effect is increased production of reactive oxygen molecules (that in turn affect cellular processes) leading to an increase in cell death (Jonassen *et al.*, 2004). Increased urinary volume is an important tool in the prevention of calcium renal stones. Urine dilution reduces crystallization phenomenon induced *in vitro* by an oxalate load in

both calcium stone formers and normal subjects (Guerra *et al.*, 2005). Uric acid stones occur especially in patients with very low urine pH (below pH 5.0) and in those with hyperuricosuria. Uric acid is very insoluble in urine at pH 5.0, but becomes significantly more soluble in urine at pH 7.0 (Sakhaee *et al.*, 2002).

The initial step in the pathogenesis of urolithiasis is precipitation of an organic matrix of mucoproteins. An important factor in this process may be the activity of the urinary enzyme urokinase which would affect the level of urinary mucoprotein. A decrease is observed in urinary urokinase concentration of renal stone patients which underlines the involvement of urokinase in renal stone formation. Increased excretion of urinary enzymes like LDH, alkaline phosphatase, β-glucoronidase, γ-glutamyl transpeptidase in calculogenic rats indicates damage to proximal tubules during stone formation (Subha and Varalakshmi, 1993).

2.3.2 Theories for stone formation

Figure 2.6 depicts the pathogenesis of kidney stone. A physico-chemical theory of urolithogenisis considers urine as a supersaturated solution in which homogenous or heterogenous nucleation can lead to initial crystal formation, which can then aggregate and grow. Calcium oxalate (CaOx) is the predominant component of most stones accounting for more than 80% stones. The remaining 20% are composed of Struvite, cystine, uric acid and other stones. No single theory of pathogenesis can properly account for human kidney stones, they are too various and their formation is too complex for simple understanding. Coe *et al.*, 2010 postulated at least three pathways that lead to stone formation.

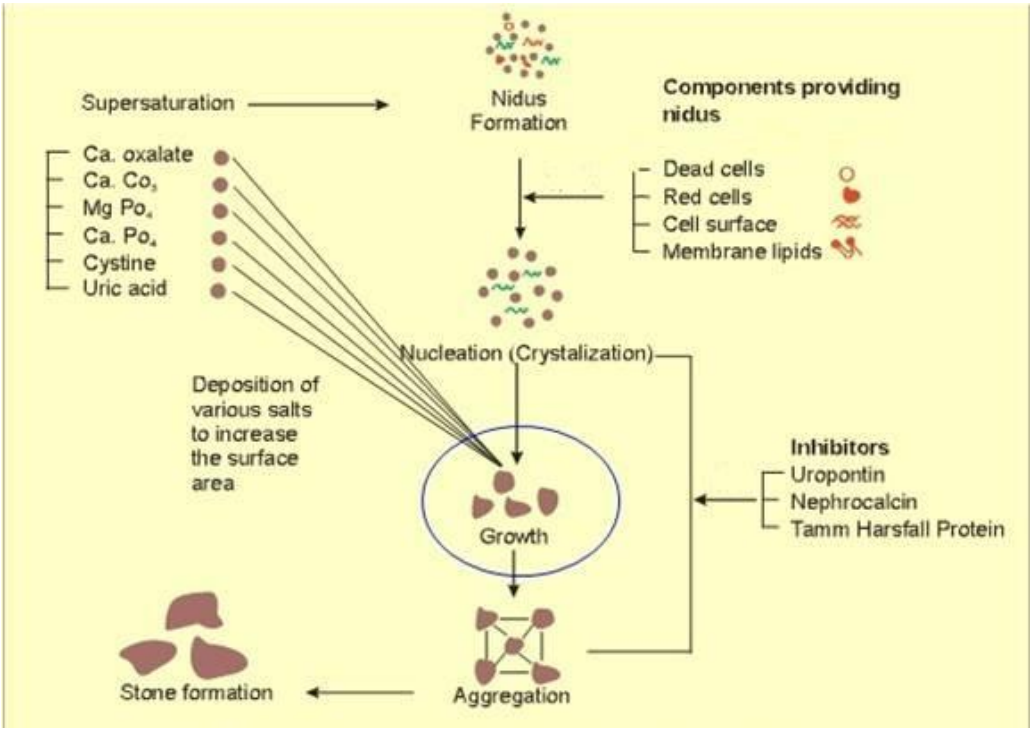


Figure 2.6: Biochemical interpretation of Urolithiasis

The first pathway is overgrowth on interstitial apatite plaque as seen in idiopathic calcium oxalate stone formers, as well as stone formers with primary hyperparathyroidism, ileostomy and small bowel resection, and in brushite stone formers. For anchoring of crystals to the urothelium, a widely held theory of Randall's plaques composed of apatite, proposes that subepithelial interstitial calcium based deposits act as a nuclei for stone formation (Johri *et al.*, 2010; Coe *et al.*, 2010). In the second pathway, there are crystal deposits in renal tubules that were seen in all stone forming groups except the idiopathic calcium oxalate stone formers. The third pathway is free solution crystallization (Coe *et al.*, 2010). Crystalluria is common while only pathological changes in kidney like renal injury may be by reactive oxygen ions or dysfunction can accomplish crystal retention and thus calculi formation (Khan, 2006).

2.3.3 Role of promoters/inhibitors in stone formation

Difference is due to the presence of urinary inhibitors that alter the process of crystallization and consequently prevent the development of stone (Atmani, 2003). Low molecular weight inhibitors include pyrophosphate and citrate while glycosaminoglycans (GAGs), nephrocalcin and Tamm horsfall protein (THP) are few examples of high molecular weight urine inhibitors (Menon and Koul, 1992). Urinary pH has an essential role in many inhibitor or promoter reactions. Most inhibitors are anionic and seem to exert their effect by binding to a calcium oxalate surface (Bihl and Meyers, 2001). The final products may be very similar though, the ways of creation are different.

Pyrophosphate was first molecule to be identified as inhibitor of crystallization. Citrate is one of the most important inhibitors of CaOx stone formation, endogenously found in urine. Magnesium, as citrate, can bind and form soluble complexes with oxalate reducing supersaturation and decreasing oxalate ion concentration (Atmani, 2003). Nephrocalcin is an acidic protein of renal tubular origin inhibiting CaOx aggregation and calcium phosphate crystallization. THP is the most abundant protein in urine and is synthesised and secreted by epithelial cells of thick ascending limb of loop of Henle and early distal convoluted tubule. It is presented on the surface and hence, affects the aggregation of the preformed crystals (Bihl and Meyer, 2001). THP can change from an inhibitor to a promoter of aggregation by polymerization. This polymerization is favoured by a highly concentrated urine, high urinary calcium and low pH (Baumann, 1998). GAGs are shown to act as inhibitor of CaOx crystal growth and aggregation by blocking growth sites (Dussol and Berland, 1996). There are certain proteins that are incorporated in the stone matrix and known to play an

inhibitory role. Prothrombin fragment 1, a peptide generated from the cleavage of prothrombin by factor Xa and thrombin, inhibits CaOx crystal aggregation and growth in rats. Inter α inhibitor has been reported to inhibit CaOx crystallization (Bihl and Meyer, 2001). Recently, a protein, human phosphate cytidylyltransferase 1 has been isolated from the human renal stone matrix in our lab and this protein is found to have CaOx growth inhibitory potency (Priyadarshini *et al.* 2009).

2.4 Diagnosis

The diagnosis of the urinary tract calculi begins with a focused history. Key elements include past or family history of calculi, duration and evolution of symptoms.

2.4.1 Urinalysis

Urinalysis should be performed in all patients with suspected calculi. Aside from typical microhematuria, important findings to note are the urine pH and the presence of crystals, which may help to identify the stone composition.

2.4.2 Abdominal ultrasonography

Although ultrasonography is readily available, quickly performed and sensitive to renal calculi, it is virtually blind to ureteral stones (sensitivity: 19%), which are far more likely to be symptomatic than renal calculi (Yilmaz *et al.*, 1998).

2.4.3 Plain-Film Radiography

Plain-film radiography of the kidneys, ureters and bladder (KUB) may be sufficient to document the size and location of radiopaque urinary calculi. Stones that contain calcium, such as calcium oxalate and calcium phosphate stones are easiest to detect by radiography. Less radiopaque calculi, such as pure uric acid stones and stones composed mainly of cystine or magnesium ammonium phosphate, may be difficult, if not impossible, to detect on plain-film radiographs. Although 90% of urinary calculi

have historically been considered to be radiopaque, the sensitivity and specificity of KUB radiography alone remain poor (Levine *et al.*, 1997). KUB radiographs are useful in the initial evaluation of patients with known stone disease and in following the course of patients with known radiopaque stones.

2.4.4 Intravenous Pyelography

Intravenous pyelography has been considered the standard imaging modality for urinary tract calculi. The intravenous pyelogram provides useful information about the stone (size, location, radiodensity) and its environment (calyceal anatomy, degree of obstruction), as well as the contra lateral renal unit (function, anomalies). Compared with abdominal ultrasonography and kidney ureter bladder (KUB) radiography, intravenous pyelography has greater sensitivity (64 to 87%) and specificity (92 to 94%) for the detection of renal calculi (Niall *et al.*, 1999). The contrast media used in intravenous pyelography carry the potential for adverse effects. Foremost is their well-documented nephrotoxic effect (Katzberg, 1997).

2.4.5 Noncontrast Helical CT

Noncontrast helical CT is being used increasingly in the initial assessment of renal colic (Smith *et al.*, 1999). This imaging modality is fast and accurate, and it readily identifies all stone types in all locations. Its sensitivity (95 to 100%) and specificity (94 to 96%) suggest that it may definitively exclude stones in patients with abdominal pain (Chen and Zagoria, 1999).

2.5 Medical Management

There are three levels of treatment that should be considered; the first constitutes general advice about dietary and drinking habits. Such advice is beneficial to all patients with calcium stone disease. The second constitutes specific dietary recommendations and/or drinking advice that should be based on the findings in the risk analysis. The aim of these measures is to lower the ion activity product below the risk level without adding pharmacological treatment. The third level always includes pharmacological treatment and is entered when the measures at level two have failed or when it is obvious that other reasons for pharmacological treatment are necessary. There is much interest among physicians and patients to identify effective measures to promote stone passage, stone dissolution and stone prevention. In the last 2 decades, the treatment of urolithiasis has changed dramatically. Management of stone disease depends on the size and location of the stones. Despite the major technical achievements for stone removal, the problem of recurrence persists. The recurrence rate of kidney stones is ~15% in the first year and as high as 50% within 5 years of initial stone (Tiselius, 2003). Effective kidney stone prevention is dependent on the stone type and the identification of the risk factors for stone formation. Recurrent stone formers usually benefit from a combination of pharmacological, dietary and drinking therapy. Table 2.3 enlists the risk factors for the stone formation and their therapeutic interventions.

Risk Factors	Dietary intervention	Drug intervention
Low urine volume	Drink >2.5L per day	
Hypercalciuria	Salt restriction, Protein moderation, High potassium diet	Hydrochlorthiazide, Chloralalidone, Indapamide, Potassium citrate
Hypocitraturia	Protein moderation, Lemon juice	Potassium citrate
Hyperoxaluria	Oxalate restriction, High calcium intake	Pyridoxine (Primary hyperoxaluria), Oxabsorb
Hyperuricosuria	Animal protein restriction	Allopurinol
Low urine pH	Animal protein restriction	Potassium citrate
Cystinuria	Drink >3L per day, Salt restriction	Potassium citrate, d-penicillamine, alphamercaptopropionylglycine (Tiopronine)
Urinary tract infection		Antibiotics

Table 2.3: Risk Factors and therapeutic interventions

Regardless of the underlying etiology of stone disease, patients should increase the fluid intake as this would reduce urinary saturation of stone forming calcium salts and dilutes promoters of CaOx crystallization (Tiselius, 2003). Given that BMI is a risk factor for renal stones and its association with diabetes and metabolic syndromes, weight loss should be advocated (Sayer *et al.*, 2010). High sodium intake increases the stone risk by reducing renal tubular calcium reabsorption and increase urinary

calcium (Butterweck and Khan, 2009). Protein confers acid and other possible lithogenic factors. The effect is more pronounced for animal *versus* plant proteins due to higher protein content per gram animal protein. Protein restriction reduces hypercalciuria via effects on multiple organs and raises urinary citrate by acting on renal proximal tubules. The rise in urinary pH and reduction in uric acid production from reduced intake of animal protein will also benefit uric acid stone formers (Moe, 2006).

Administration of thiazides has a pronounced and well documented effect in reducing urinary calcium though; long term usage is limited due to side effects like fatigue, dizziness, impotence, musculoskeletal symptoms or gastrointestinal complications. Another complication is thaizide induced potassium depletion which causes intracellular acidosis and can lead to hypokalemia and hypocitraturia. Hence, these should always be used in conjugation with low sodium intake or high potassium diet or potassium citrate (Heilberg and Schor, 2006; Moe, 2006; Atmani, 2003; Sayer *et al.*, 2010).

Potassium citrate reduces urinary saturation of calcium salts and is an effective agent for uric acid stones. However, adverse effects of gastrointestinal origin including epigastric pain, abdominal distention or diarrhoea are common (Heilberg and Schor, 2006; Moe, 2006). Citrate inhibits CaOx and calcium phosphate crystallization by lowering urinary ionized calcium, thereby reducing urinary saturation of stone forming calcium salts and by preventing nucleation, crystallization and agglomeration of calcium salts (Tracy and Pearle, 2009). Orange juice and lemonade have been shown to increase urinary citrate and observational studies suggest this to be a reason for low occurrence of stone in lemonade drinkers (Kang *et al.*, 2007).

Dietary oxalate restriction is more difficult and needs good counselling because oxalate rich foods are more variable and ubiquitous. Increase in dietary oxalate can substantially alter excreted oxalate in urine. Pyridoxine, which promotes the conversion of glyoxylate to glycine, is the only medicine available to treat primary hyperoxaluria (Moe, 2006). The excessive intestinal absorption of oxalate in patients with enteric hyperoxaluria can be treated by restricted intake of oxalate rich products, administration of oxabsorb, a marine hydro-colloid that forms complexes with oxalate and calcium supplements (Tiselius, 2003). The use of probiotic (colonisation with oxalate consuming bacteria, *Oxalobacter formigenes*) could lead to decreased intestinal absorption of oxalate and a corresponding decrease in urinary CaOx. Although, the reduced urinary oxalate levels and low side effects profile associated with this probiotic are promising, prospective trials must be completed to confirm findings in stone formers (Tracy and Pearle, 2009).

Allopurinol blocks uric acid production, reducing heterogenous nucleation of CaOx by both uric acid and monosodium urate. In addition, the adsorption of normally occurring macromolecular inhibitors of CaOx crystallization by uric acid or monosodium urate could possibly be averted when using this drug (Heilberg and Schor, 2006). It is also proposed that allopurinol might have an antioxidant role in nephrons, thus counteracting the negative effects of free radicals (Tiselius, 2003). In case of high doses, allopurinol may conduct to the formation of xanthine and oxypurinol calculi (Atmani, 2003).

Stones less than 5mm 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 as shown in Figure 2.7 (Gurocak and Kupeli, 2006). ESWL produces high pressure shock waves which causes the stone to be stressed, then fractured and eventually disintegrated. 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. In addition to ESWL, URS is used for the removal of ureteral stones. The new generation uteroscopes are flexible, smaller in diameter, stiffer and more durable and have an improved tip deflection. The major drawback of URS is that it is more invasive than ESWL and the rate of ureteral perforation and stricture formation remains 2 to 4%. In contrast, the major advantage of URS is that it is cheaper and results in higher and faster stone free rates (Butterweck and Khan, 2009). PCNL is recommended when the stone is quite large or in a location that does not allow effective use of ESWL. In this procedure, tiny incision in the back is made directing to the kidney, where nephroscope is used to break and remove stone. The advantage of PCNL over ESWL is that stone fragments are removed by the surgeon rather than relying on their natural passage (Tandon *et al.*, 2006). It remains unclear which treatment modality is better than the other and the final decision should be based on the patient's preference, on the size and location of stone, expertise of the physician and the costs of the procedure.

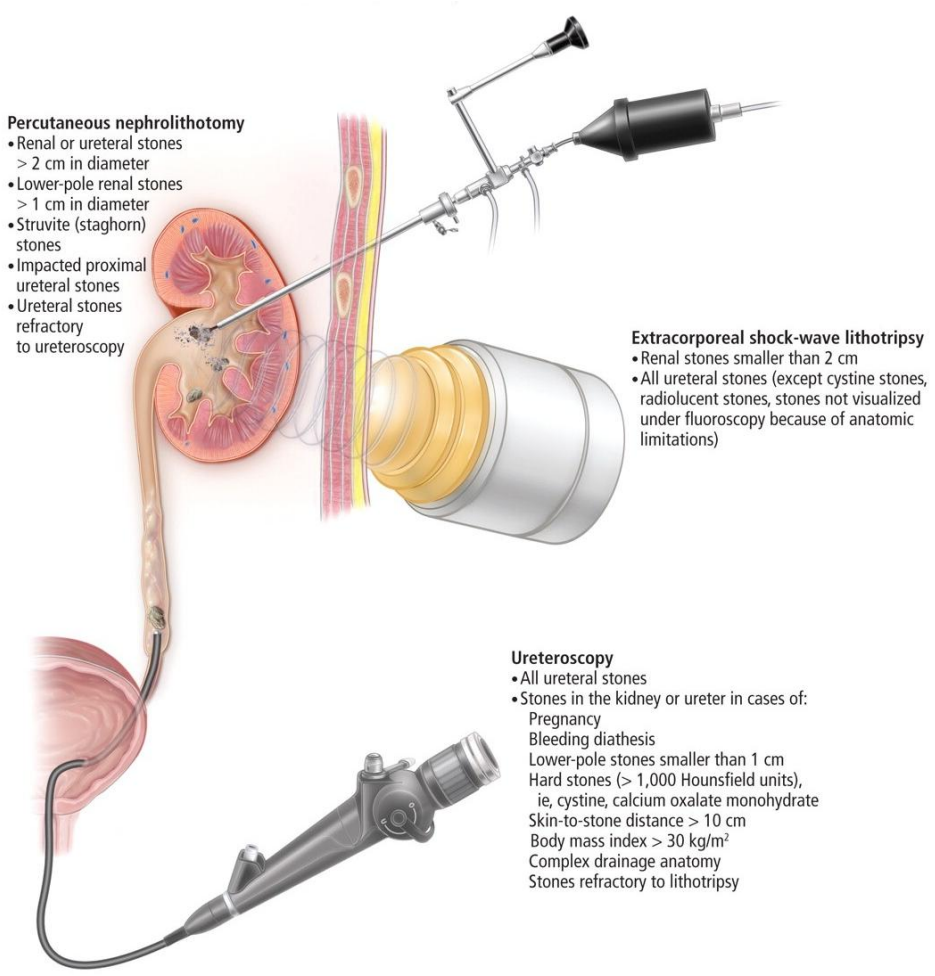


Figure 2.7: Urinary Stones: Choice of surgical intervention

Though, 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 persistent of recurrence remain as problems to overcome. Thus, an adjunct to these conventional methods as phytotherapy is highly recommended.

2.6 Phytotherapy

2.6.1 Social and economic impacts

Recently, there has been a shift in universal trend from synthetic to herbal medicines. 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. It has been confirmed by WHO that, approximately 75% of the global population, most in the developing world, depends on botanical medicines for their basic healthcare needs (Verma and Singh, 2008; Atmani, 2003). About 500 plants with medicinal use are reported in ancient literature and around 800 plants have been used in the indigenous systems of medicine (Verma and Singh, 2008). Substances first isolated from the plants account for approx 25% of the western pharmacopoeia, with another 25% derived from the modification of chemicals first found in natural products (Atmani, 2003).

In India, around 20,000 medicinal plant species have been recorded recently, but more than 500 traditional communities use about 800 plants for curing different diseases (Kamboj, 2000). The use of herbal medicine is becoming popular due to toxicity and side effects of allopathic medicines. Many remedies have been employed during the ages to treat urinary stones. In the traditional system of medicine, most of the remedies were taken from plants and they proved to be useful though the rationale behind their use is not well established through systematic pharmacological and clinical studies except for some composite herbal drugs.

The marketed composite herbal formulations, Cystone (Himalaya Drug Company, India), Calcuri (Charak Pharmaceuticals, Mumbai, India), Chandraprabhabati (Baidyanath, India), Neeri (Aimil Pharmaceitucals, India), Uriflow (Bioneutrix Labs)

and Culdisol (Ganga Pharmaceuticals, India) have been used worldwide to dissolve urinary calculi in kidney and urinary bladder.

2.6.2 Scientific Evidences

Concerning herbal medicines, there is a large number of plant species described in many pharmacopoeia in the world as remedies for urolithiasis. However, few investigators have devoted their efforts to study these plants in order to elucidate the mechanism by which these plants exert their effect as shown in Figure 2.8 and identify active biomolecules. Table 2.4 lists the phytotherapeutic agents scientifically evaluated till date by various researchers.

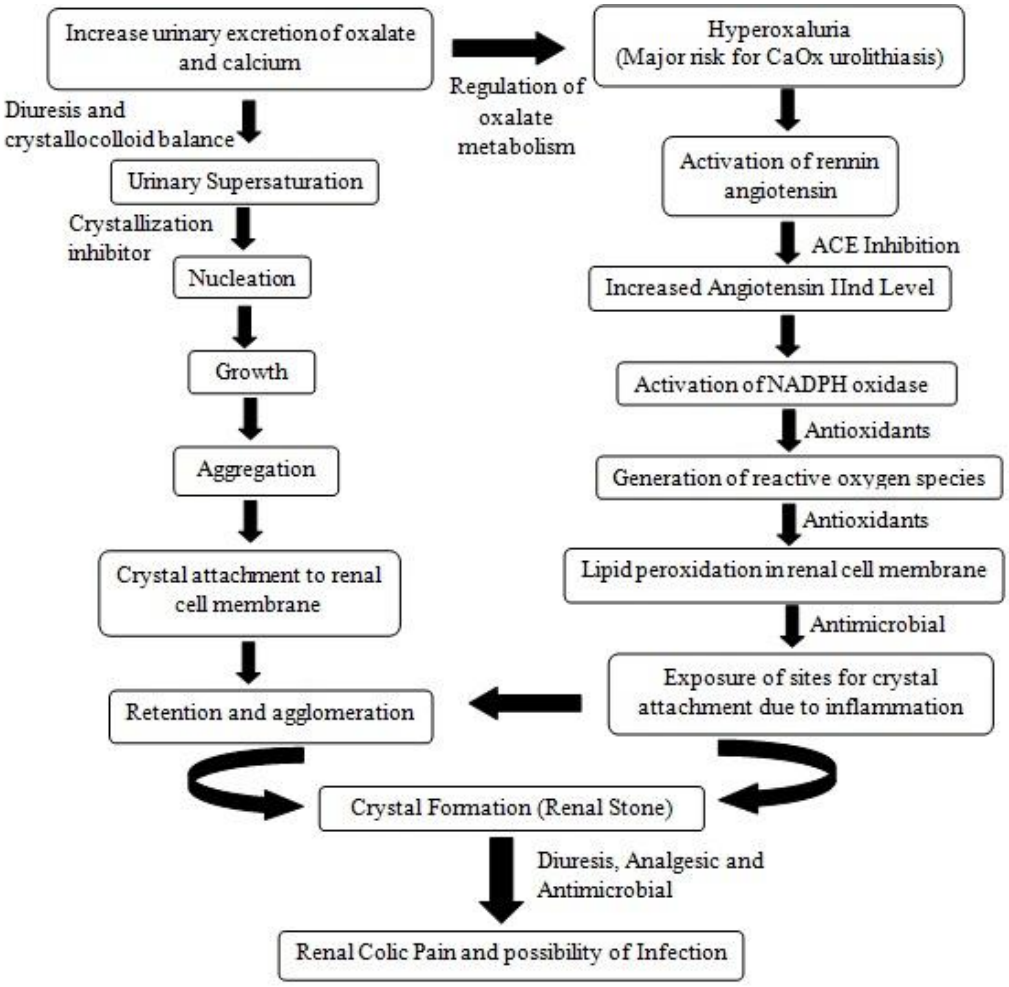


Figure 2.8: Mechanism of action of phytotherapeutic agents

Phytotherapeutic Agent	Type of study	References
Herniaria hirsuta	*In vitro*, cell lines, *in vivo* (animals)	Atmani *et al.* 2003, Atmani *et al.* 2004
Phyllanthus niruri	Cell lines, *in vivo* (animals), *in vivo* (humans)	Micali *et al.* 2006, Boim *et al.* 2010, Nishiura *et al.* 2006, Barros *et al.* 2003, Barros *et al.* 2006, Frietas *et al.* 2002, Campos, and Schor 1999
Bergenia lingulata	*In vitro*, *in vivo* (animals)	Garimella *et al.* 2001, Bashir and Gilani 2009
Dolichos biflorus	*In vitro*, *in vivo* (animals)	Garimella *et al.* 2001, Peshin and Singla 1994, Schwartz *et al.* 2000, Singh *et al.* 2010, Bijarnia *et al.* 2009
Tribulus terrestris	*In vitro*, *in vivo* (animals)	Sangeeta *et al.* 1994, Anand *et al.* 1994, Joshi *et al.*, 2005a, 2005b
Ammi visnaga	Cell lines, *in vivo* (animals)	Vanachayangkul *et al.* 2010, 2011
Aerva lanata	*In vivo* (animals)	Soundarajan *et al.* 2006, Shirwaikar *et al.* 2004
Aerva lanata and *Vediuppu chunam*	*In vivo* (animals)	Selvam *et al.* 2001
Pomegranate juice	*In vivo* (animals)	Tugcu *et al.* 2008
Lemon juice	*In vitro*, *in vivo* (animals)	Touhami *et al.* 2007, Oussama *et al.* 2005
Cranberry juice	*In vivo* (animals)	McHarg *et al.* 2003
Grapefruit juice	*In vivo* (animals)	Goldfarb and Asplin 2001
Cyclea peltata	*In vivo* (animals)	Christina *et al.* 2002
Rotula aquatic	*In vitro*, *in vivo* (animals)	Christina *et al.* 2002, Prasad *et al.* 1993
Raphanus sativus	*In vivo* (animals)	Vargas *et al.* 1999
Zea mays	*In vivo* (animals)	Grases *et al.* 1993
Costus spiralis	*In vivo* (animals)	Araujo *et al.* 1999
Agropyren repens	*In vivo* (animals)	Grases *et al.* 1995

Rosa canina	*In vivo* (animals)	Grases *et al.* 1995
Paronychia argentea	*In vivo* (animals)	Bouanani *et al.* 2010
Andrographis paniculata	*In vivo* (human)	Maungman *et al.* 1995
Orthosiphon stamineus	*In vivo* (animals)	Arafat *et al.* 2008, Adam *et al.* 2009
Orthosiphon grandiflorus	*In vivo* (animals)	Premgamone *et al.* 2001
Crataeva nurvala	*In vivo* (animals)	Varalakshmi *et al.* 1990, Singh and Kapoor, 1991
Kampou medicine	*In vivo* (animals)	Koide *et al.* 1995
Banana stem	*In vivo* (animals)	Poonguzhali and Chegu 1994
Tamarindus indicus	*In vivo* (humans)	Rathore *et al.* 1993
Trachyspermum ammi	*In vitro, in vivo* (animals)	Kaur *et al.* 2009, Kaur *et al.* 2009
Trigonella foenum-graceum	*In vivo* (animals)	Ahsan *et al.* 1989, Laroubi *et al.* 2007
Mimosa pudica	*In vivo* (animals)	Joyamma *et al.* 1990
Asparagus racemosus	*In vivo* (animals)	Christina *et al.* 2005
Plecranthus amboinicus	*In vivo* (animals)	Jose *et al.* 2005
Moringa olifera	*In vivo* (animals)	Karade *et al.* 2006
Ammannia baccifera	*In vivo* (animals)	Prasad *et al.* 1994
Quercus salicina Blume	Cell lines, *in vivo* (animals)	Moriyama *et al.* 2007, 2009
Green tea	Cell lines, *in vivo* (animals)	Itoh *et al.*, 2005, Jeong *et al.* 2006
Rubia cordifolia	*In vivo* (animals)	Divakar *et al.* 2010
Cynodon dactylon	*In vivo* (animals)	Atmani *et al.* 2009
Sesbania grandiflora	*In vivo* (animals)	Doddola *et al.* 2008
Nigella sativa	*In vivo* (animals)	Hadjzadeh *et al.* 2007
Berberis vulgaris	*In vivo* (animals)	Bashir *et al.* 2010
Terminalia arjuna	*In vitro*	Chaudhary *et al.* 2010
Achyranthes aspera	*In vitro*, cell lines	Aggarwal *et al.* 2010

Table 2.4: Various phytotherapeutic agents used in the treatment of urolithiasis

Herniaria hirsuta, one of the most widely used herbal remedy, is a member of Caryophyllaceae, majorly found in Morocco. Atmani *et al.* reported that *H. hirsuta* progressively decreased the adhesion of CaOx crystals to canine kidney cells and it didn't appear to adversely affect cell growth under conditions in their study. Prior exposure of crystals but not cells to the extract, blocked crystal binding suggesting that plant molecule can coat and exert their efficacy at crystal surface. Increased membrane fluidity correlates positively with crystal adhesion to the cells and *Herniaria* extract was able to alter crystal adhesion in increased fluidity. In the *in vivo* analysis with *Herniaria* decoction, there was a decrease in urinary oxalate in comparison to nephrolithiatic rats. The extract also decreased the size of the crystals though their number was increased in urine and the examination of the kidney revealed less deposition of CaOx crystals in treated rats. Crystalluria is not the only risk factor in urinary stone disease because it can be observed in normal individuals. Crystal size seems to be more effective in stone formation because larger crystals carry a higher risk of retention in the urinary tract. Moreover, herb extract resulted in higher calcium oxalate dihydrate excretion than calcium oxalate monohydrate excretion (Atmani *et al.*, 2003; Atmani *et al.* 2004). This may be considered another antilithogenic effect of the extract because calcium oxalate dihydrate crystals bind less tightly to the epithelial cells (Wesson *et al.*, 1998; Atmani *et al.*, 2000)

Another important plant studied for its antilithiatic potency is *Phyllanthus niruri*, used majorly in Brazilian folk medicine. The clinical beneficial effects of *Phyllanthus* may be related to the ureteral relaxation, helping to eliminate calculi or to clear fragments following lithotripsy (Micali *et al.*, 2006) or also to a putative reduction of the excretion of urinary crystallization promoters such as calcium (Boim *et al.*, 2010;

Nishiura *et al.*, 2006). Barros *et al.* suggested that *Phyllanthus niruri* interfered with the arrangement of precipitating crystals, probably by modifying the crystal-crystal and/or crystal-matrix interaction (Barros *et al.*, 2003; Barros *et al.*, 2006). *Phyllanthus* has an inhibitory effect on the crystal growth, independent of changes in urinary excretion of citrate and magnesium, but might be related to higher incorporation of glycosaminoglycans (GAGs) into the calculi (Frietas *et al.*, 2002). The aqueous extract of *Phyllanthus* exhibited a potent and effective non concentration dependent inhibitory effect on CaOx crystal internalization by MDCK cells. This response was present even at very high (pathologic) CaOx concentration and no *Phyllanthus* induced toxicity could be detected (Campos and Schor, 1999).

Bergenia lingulata and *Dolichos biflorus* are 2 herbal remedies used in folklore. Garimella *et al.* compared different forms of these extracts with results in a control group. They measured urine calcium using titrimetry and urine phosphorus using colorimetric analysis. As a result, they noted that the detannated and deproteinised extract of *Berginia lingulata* was more active than the whole extract. In addition, *Dolichos biflorus* showed activity comparable to that of the marketed formulation for decreasing calcium and phosphate precipitation. The investigators concluded that *Dolichos* is more active as a prophylactic agent than *Berginia lingulata* for the urinary calculi (Garimella *et al.*, 2001). *In vitro* effect of *D. biflorus* seeds on crystallization on crystallisation of CaP was undertaken by Peshin *et al.* The results suggested that the inhibitors in seeds were water soluble, heat stable, polar, non-tannin and non protein in nature. Though in the same study two or more different inhibitors of CaP were reported since both dialysate and dialysed fraction contained inhibitory activity (Peshin and Singla, 1994). Schwartz and colleagues demonstrated the utility of *D.*

biflorus through a prospective trial evaluating 24hr urine samples. No statistical significant results were obtained though there was an increase in urinary magnesium providing mechanism of action as magnesium is an inhibitor of stone formation (Schwartz *et al.*, 2000). Kulattha can be used to reduce the recurrence of CaOx stones and it is shown to have better results than the use of conventional potassium citrate in such patients by measuring the size of renal calculi (Singh *et al.*, 2010). Bashir suggested the mechanism of the antiurolithiatic activity in *Berginia* mediated possibly through CaOx crystal inhibition, diuretic, hypermagnesuria and antioxidant effects (Bashir and Gilani, 2009).

Ammi visnaga, a popular Saudi folk medicine, is an example of herb whose antilithiatic effect is attributed to its diuretic activity in maintaining the oxalate concentration below the supersaturation level and amelioration of uremia and hyperbilirubinemia (Khan *et al.*, 2001). *A. visnaga* was evaluated for its management of kidney stones in comparison to khellin and visnagin in renal epithelial cells, LLC-PK1 and MDCK and *in vivo* model too. Studies revealed that *Ammi visnaga* extract, khellin and visnagin are cytoprotective and are beneficial in management of kidney stone disease (Vanachayangkul *et al.*, 2010; Vanachayangkul *et al.*, 2011).

The efficacy of two siddha drugs, *Aerva lanata* and *Vediuppu chunam* as antilithic agents, were studied in rats. Increased urinary excretion of calcium, oxalate, uric acid, phosphorus and protein in hyperoxaluric rats was brought down significantly by administration of *A. lanata* and *V. chunam*. Decreased magnesium excretion in hyperoxaluric rats was also normalized. Combination therapy was more effective in management of kidney stones (Selvam *et al.*, 2001). Administration of *A. lanata* aqueous suspension to CaOx urolithic rats reduced oxalate synthesizing enzymes,

diminished markers of crystal deposition in kidney (Soundarajan *et al.*, 2006). Ethanolic extract of *A. lanata* possess marked nephroprotective activity with minimal toxicity (Shirwaikar *et al.*, 2004).

Juices are said to be a major source of citrate, an inhibitor of calculi formation. Pomegranate juice (PJ) shows a protective effect in the ethylene glycol (EG) induced crystal depositions in renal tubules. There was limited or no crystal formation in EG+PJ treated groups. There was a marked inducible nitric oxide synthase (iNOS) and p65 expression only in EG given rats compared to PJ treated grups, emphasising the antioxidant role of pomegranate juice (Tugcu *et al.*, 2008). The EG/NH4Cl induced increase in kidney calcium levels was inhibited by administration of lemon juice. There was also a decrease in CaOx crystal deposition on administering lemon juice to hyperoxaluric rats (Touhami *et al.*, 2007). In the synthetic urine too, the inhibition rate of CaOx crystallisation increased gradually with lemon juice concentration (Oussama *et al.*, 2005). Cranberry juice is a popular herb and McHarg *et al.* investigated the antilithogenic effect of this juice. They found increased urinary citrate excretion together with decreased urinary excretion of oxalate and calcium ions *in vivo* (Mc Harg T *et al.*, 2003). Grapefruit juice is another widely studied herb for urinary stone disease. The studies reveal that this juice increased the urinary excretion of citrate and magnesium (Goldfarb and Asplin, 2001). Citrus fruits could represent a natural alternative to potassium citrate in the management of nephrolithiasis because it is better tolerated and cost effective too (Trinchieri *et al.*, 2002).

Cyclea peltata, which belongs to the Menispermaceae family, is another example of commonly used herbs for stone disease in Kerala, a state in south India. Christina *et al.* investigated the effect of this herb on stone formation induced by ethylene glycol.

It was shown that simultaneous administration of this powder resulted in decreased urinary calcium and oxalate while serum magnesium was increased, favouring the antilithogenic effect of this remedy (Christina *et al.,* 2002). *Rotula aquatica* lour shares a similar mechanism of action and simultaneous treatment with this remedy decreased the calcium and oxalate ion concentration in urine, thus confirming the stone inhibitory effect (Christina *et al.*, 2002). Ethyl acetate extract *Rotula aquatica* showed significant antilithiatic activity against Struvite stones and CaOx stones (Prasad *et al.*, 1993). *Raphanus sativus* is a plant of European origin, known for its medicinal properties. Vargas *et al.* performed a study in which they induced stone formation by implanting a zinc disc in the bladder of rats. Aqueous extract showed to inhibit calculus formation in a dose dependent manner. The observed diuretic activity seems to be the dominant effective mechanism responsible for preventing calculus deposits (Vargas *et al.*, 1999). *Zea mays* has also been described to be diuretic with no influence on important urinary risk factors such as citruria, calciuria or urinary pH (Grases *et al.*, 1993). *Costus spiralis* is a popular herbal remedy of Brazilian folk medicine with an unknown mechanism of action. Araujo *et al.* investigated this plant by implanting a foreign body in the bladder of adult rats and concluded that this plant decreased the growth of urinary stones, which supports the folk information but this antilithogenic effect of *Costus spiralis* was not related to its diuretic activity of the plant (Araujo *et al.*, 1999). *Agropyron repens* is a widely consumed extract for nephritis, urethritis and urinary calculi and it has been thought to exert antimicrobial features which might be responsible for its protective effect (Grases *et al.*, 1995). Grases *et al.* also reported that, another plant, *Rosa canina*, increased urinary citrate excretion, a remarked urinary stone stone inhibitor (Grases *et al.*, 1992). Renal

protection and antiurolithiatic effects of aqueous extract and butanolic extract of *Paronychia argentea*, a traditional Algerian plant commonly called as Algerian tea was compared. Administration of the butanolic extract of aerial parts to rats with sodium oxalate induced lithiasis reduced and prevented the growth of urinary stones (Bouanani *et al.*, 2010)

Another antilithogenic effect of some herbal remedies is attributed to the antimicrobial activity. In regard to this aspect, Maungman *et al.* evaluated *Andrographis paniculata*, an eastern herb, for its bacteriostatic activity. They prescribed this drug in patients who underwent ESWL and noted a significant decrease in pyuria and hematuria (Muangman *et al.*, 1995).

Orthosiphon has been used for the treatment of kidney and bladder stones and urinary tract infections. The proposed active ingredients are flavonoids that are believed to have diuretic, antiseptic and litholytic properties. Arafat and colleagues demonstrated not only the diuretic effect of *Orthosiphon stamineus* extract in rats but also an increase in sodium excretion (Arafat *et al.*, 2008; Adam *et al.*, 2009). A study was performed to compare the efficacy of *Orthosiphon grandiflorus* and the drug sodium potassium citrate (SPC) in the treatment of renal calculi. The study indicates that treatment of renal calculi with *O. grandiflorus* tea is an alternative means of management with lesser side effects (Premgamone *et al.*, 2001).

The use of *Crataeva nurvala* as a treatment for urolithiasis dates to 210 BC. Varalakshmi and colleagues helped delineate its mechanism of action. Simultaneous administration of *C. nurvala* extract reduced both GAO activity and renal oxalate crystallization. There was also elevated urinary magnesium (Varalakshmi *et al.*, 1990). Studies reveal that ethanolic extract has a dose related antiurolithiatic activity

in albino rats induced by foreign body method using glass beads (Singh and Kapoor, 1991).

Several traditional Chinese medicines or plants that are used in Kampou medicine also have demonstrated their abilities to inhibit CaOx crystallization (Koide *et al.*, 1995). To ascertain the beneficial effects of banana stem extract on urinary risk factors, a prospective study showed that the plant extract reduced significantly urinary oxalate in experimentally hyperoxaluric rats (Poonguzhali and Chegu, 1994). *Tamarindus indicus* was studied in normal subjects and in stone formers. Its intake showed significant beneficial effect in inhibiting spontaneous crystallization in both normal subjects and in stone formers (Rathore *et al.*, 1993). Antiurolithiatic activity of some Saudi Arabian folklore plants viz. *Trachyspermum ammi*, *Ammi majus* and *Trigonella foenum-graceum* was evaluated. *Trigonella foenum-graceum* was found to reduce CaOx urolithiasis (Ahsan *et al.*, 1989; Laroubi *et al.*, 2007).

Mimosa pudica was not very effective in either preventing stone deposition or dissolving preformed stones (Joyamma *et al.*, 1990). The ethanolic extract of *Asparagus racemosus* had an inhibitory potential on lithiasis induced by oral administration, by significantly reducing the elevated levels of calculogenic ions in urine and increasing the urinary concentration of magnesium (Christina *et al.*, 2005). The fresh juice of leaves of *Plectranthus amboinicus* lour has an effect against renal calculi, particularly of CaOx origin (Jose *et al.*, 2005). The aqueous and ethanolic extracts of the root wood of *Moringa olifera* Lam significantly reduced the elevated urinary oxalate, showing a regulatory action on endogenous oxalate synthesis in hyperoxaluria induced with ethylene glycol (Karade *et al.*, 2006). Ethanolic extract of

Ammannia baccifera was found to be effective as prophylactic and curative against phosphate type stones (Prasad *et al.*, 1994).

Oxalate, has been shown to exert cytotoxic effects on renal tubular epithelial cells, attributable to increased oxidative stress within the cells. It was reported, that an extract prepared from *Quercus salicina* Blume/ *Quercus stenophylla* Makino could suppress cell injury induced by oxalate exposure, by scavenging free radicals and suppressing the activation of NADPH oxidase (Moriyama *et al.*, 2007). The group established the antiurolithiatic potency of the same in the rat urolithiatic model too (Moriyama *et al.*, 2009). Similar effects were reported for epigallocatechin gallate (EGCG) from green tea, which also inhibited free radical production from oxalate (Jeong *et al.*, 2006). Hydro-alcoholic extract of *Rubia cordifolia* Linn. supplementation protects against EG induced urolithiasis as it reduces and prevents growth of urinary stones. The mechanism underlying this effect is mediated through an antioxidant, nephroprotection and its effect on the urinary concentration of stone forming constituents and risk factors (Divakar *et al.*, 2010). Atmani *et al.* evaluated the effect of *Cynodon dactylon* as preventive and curative agent in experimentally induced nephrolithiasis in rat model. There was a decrease in CaOx deposition in medullary and papillary sections (Atmani *et al.*, 2009). *Sesbania granidiflora* leaf juice showed significant antiurolithiatic potency against CaOx stones and also exhibited antioxidant properties (Doddola *et al.*, 2008). Treatment of rats with ethanolic extract of *Nigella sativa* seeds reduced the number of CaOx deposits and also reduced the urine concentration of CaOx (Hadjzadeh *et al.*, 2007). Aqueous methanolic extract of *Berberis vulgaris* administration to hyperoxaluric rats inhibited CaOx crystal deposition in renal tubules and protected against associated changes

including polyuria, weight loss, impaired renal function and development of oxidative stress in kidneys (Bashir *et al.*, 2010).

2.6.3 Proteins: Potent antiurolithiatic biomolecules

Studies were carried out on various Indian medicinal plants in our laboratory. The plants studied were *Dolichos biflorus, Trachyspermum ammi, Tamarindus indicus, Terminalia arjuna, Achyranthes aspera*. The antiurolithiatic potency was carried out *in vitro* and *in vivo*. Till date, the plants tested were found to be inhibitory towards calcareous stones including both calcium oxalate and calcium phosphate. The protein biomolecules isolated from the plants were significantly found to effective antiurolithiatic agents *in vitro* and *in vivo.*

Our group isolated and purified a novel dimeric antilithiatic protein (98kDa) from the seeds of *Dolichos biflorus*, which was able to inhibit CaOx crystallization *in vitro* (Bijarnia *et al.*, 2009) and *in vivo* (unpublished data). Amino acid analysis showed abundant acidic amino acids in this protein. Aspartic acid and Glutamic acid are known to interact with the positively charged calcium, thus, making it unavailable to bind with oxalate. This protein presented sequence similarity with a calcium binding protein, calnexin of *Pisum sativum* (Bijarnia *et al.*, 2009). A novel CaOx crystal growth inhibitor was purified from the seeds of *Trachyspermum ammi* by our group. Presence of 2 EF hand domains signifies its calcium binding properties, a feature of most kidney stone inhibitory properties, a feature of most kidney stone inhibitory properties (Kaur *et al.*, 2009). The antilithiatic potential of this protein was confirmed *in vivo* by its ability to maintain renal functioning, reduce renal injury and decrease crystal excretion in urine and retention in renal tissues (Kaur *et al.*, 2009). An antilithiatic protein was also isolated from *Terminalia arjuna* bark, a plant commonly

found in Himachal Pradesh, which showed promising results *in vitro.* The low molecular weight protein (~14kDa) was found to inhibit CaOx growth significantly (Chaudhary *et al.*, In Press). The activity of various extracts of *Terminalia arjuna* were tested *in vitro* on different phases of calcium phosphate and calcium oxalate crystallisation and the plant proved to be quite effective with maximum inhibition been exhibited by *n*-butanol fraction (Chaudhary *et al.*, 2010). The studies with aqueous extract of *Achyranthes aspera* indicated that besides having a cytoprotective role, it also has a potential to inhibit both nucleation and the growth of CaOx crystals *in vitro* (Aggarwal *et al.*, 2010).

2.7 *Tribulus terrestris*

Classification:

Kingdom: Plantae

Division: Angiospermae

Class: Dicotyledoneae

Order: Geraniales

Family: Zygophyllaceae

Genus: *Tribulus*

Species: *terrestris* Linn.

Common name:

English: Land-caltrops, Puncture-vine

Hindi: Gokhru,

Sanskrit: Gokshura

Part used: Dried spiny fruit

2.7.1 Botanical description

Tribulus terrestris is an annual or perennial, prostrate herb with many slender, spreading branches and silky-villous young parts. Leaves are abruptly simple, pinnate and opposite. Leaflets almost sessile, rounded or oblique at the base, mucronate at the apex, flowers bright yellow, solitary, pseudo axillary or leaf opposed. Fruits are 5 angled or winged spinous tuberculate woody schizocarp, separating into five cocci, each coccus having two long, stiff, sharp divaricate spines towards the distal half and two shorter ones nearer the base, seeds one or more in each coccus (Figure 1.2).

2.7.2 Geographical distribution

The plant grows wild throughout India; the shrub thrives in well irrigated soil up to an altitude of 3000 m.

2.7.3 Traditional uses

The roots and fruits are sweet, cooling, diuretic, aphrodisiac, emollient, appetiser, digestive, anthelmintic, expectorant, anodyne, anti-inflammatory, alterant, laxative, cardiotonic, styptic, lithotriptic and tonic. They are useful in strangury, dysuria, vitiated conditions of vata and pitta, renal and vesical calculi, anorexia, dyspepsia, helminthiasis, spermatorrhoea, anaemia, scabies, ophthalmia and general weakness. The leaves are astringent, diuretic, aphrodisiac, depurative, anthelmintic and tonic. They are useful in gonorrhoea, inflammation, menorrhagia, strangury, leprosy, skin diseases, verminosis and general weakness. The seeds are astringent, strengthening and are useful in epistaxis, haemorrhages and ulcerative stomatitis. The ash of the whole plant is good for external application in rheumatoid-arthritis (Warrier *et al.*, 1996). The diuretic properties of the plant are due to the large quantities of the nitrates present as well as the essential oil which occurs in the seeds (Nadkarni, 1993).

2.7.4 Pharmacology

Tribulus terrestris had shown chemopreventive effect skin papilloma genesis in mice (Kumar *et al*., 2006). It also showed protective effects in diabetes mellitus (Amin *et al*., 2006). *In vivo* study suggested that methanolic and aqueous extracts of *T. terrestris* having antihypertensive and vasodilator effects (Phillips *et al*., 2006). The ethanolic extract of *T. terrestris* exhibited protective effect against Cadmium-induced testicular damage *in vivo* (Rajender *et al*., 2011). In a preliminary study, the diuretic effect of *Tribulus terrestris* and *Hygrophila spinosa* water extracts in albino rats was evaluated (Kumari and Iyer, 1967; Singh *et al.*, 1991). The effect of an aqueous extract of *Tribulus terrestris* administered orally at a dose of 5 g/kg body wt and restoration in urinary oxalate was observed (Sangeeta *et al.*, 1993). Few studies were conducted to evaluate the therapeutic use of *Tribulus terrestris* in various urinary disorders including urolithiasis (Anand *et al.*, 1994; Sangeeta *et al.*, 1994).The inhibitory potency of the extract of putatively litholytic plant, *Tribulus terrestris* was tested on the growth of brushite and CaOx crystals *in vitro* and it exhibited appreciable amount of inhibition (Joshi *et al.*, 2005a; 2005b).

Tribulus terrestris is a common constituent of various marketed antiurolithiatic herbal formulations like Cystone (Himalaya Drug Company), Neeri (Aimil Pharmaceuticals), Uritone (Aimil Pharmaceuticals), Uriflow (Bioneutrix), Culdisol (Ganga Pharmaceuticals) and Culin forte (Alopa Herbal).

MATERIALS

&

METHODS

3.1 Antiurolithiatic potency of *Tribulus terrestris in vitro*

3.1.1 Preparation of the Tribulus terrestris extract

The dried and matured fruits of *Tribulus terrestris* were obtained from "Natural Remedies Pvt. Ltd." at Bangalore in India. A collection of voucher specimen is available with the company.

The air dried fine powdered plant fruits were weighed and soaked in double-distilled water overnight at room temperature. The extract was then filtered using Whatman No. 1 filter paper and the filtrate was concentrated *in vacuo* using a rotary evaporator and the residue was freeze dried (Kandil *et al.*, 1994). The final dried samples were stored in labelled sterile bottles and kept at −20°C. The various concentrations of the plant sample tested for their inhibitory potency were 25µg/ml, 50µg/ml, 100µg/ml, 200µg/ml, 400µg/ml and 1000µg/ml, prepared at the time of experiment and were referred to as aqueous extract of *Tribulus terrestris.*

3.1.2 Nucleation of Calcium oxalate (CaOx) crystals

The method used was similar to that described by Hennequin *et al.* with some minor modifications (Hennequin *et al.*, 1993). Solutions of calcium chloride ($CaCl_2$) and sodium oxalate ($Na_2C_2O_4$) were prepared at the final concentration of 3 mmol/L and 0.5 mmol/L, respectively, in a buffer containing Tris-Cl 0.05 mol/L and NaCl 0.15 mol/L at pH 6.5. Both solutions were filtered through a 0.22 µm filter; 33 ml of $CaCl_2$ solution was mixed with 3.3ml of the aqueous extract at different concentrations. Crystallization was started by adding 33 ml of $Na_2C_2O_4$ solution. The final solution was magnetically stirred at 800 rpm using a PTFE-coated stirring bar. The temperature was maintained at 37^{o}C. The absorbance of the solution was monitored at 620 nm after every 1 min.

The percentage inhibition produced by the plant extract was calculated as follows:

$$\% \text{ Relative Inhibition} = [(T_{sc}-T_{st})/T_{sc})] \text{ X } 100$$

Where, 'T_{sc}' is the turbidity slope of the control,

'T_{st}' is the turbidity slope in the presence of the test sample.

3.1.3 Growth of Calcium oxalate (CaOx) crystals

Inhibitory activity against CaOx crystal growth was measured using the seeded, solution-depletion assay described previously by Nakagawa and colleagues (Nakagawa *et al.*, 1985). Briefly, an aqueous solution of 10 mM Tris-Cl containing 90 mM NaCl was adjusted to pH 7.2 with 4 N HCl. Stone slurry (1.5 mg/ml) was prepared in 50 mM sodium acetate buffer (pH 5.7). CaOx crystal seed was added to a solution containing 1 mM calcium chloride ($CaCl_2$) and 1 mM sodium oxalate ($Na_2C_2O_4$). The reaction of $CaCl_2$ and $Na_2C_2O_4$ with crystal seed would lead to deposition of CaOx (CaC_2O_4) on the crystal surfaces, thereby decreasing free oxalate that is detectable by spectrophotometry at λ214 nm. When aqueous extract is added into this solution, depletion of free oxalate ions will decrease if the test sample inhibits CaOx crystal growth.

Rate of reduction of free oxalate was calculated using the baseline value and the value after 30-second incubation with or without test sample. The relative inhibitory activity was calculated as follows:

$$\% \text{ Relative inhibitory activity} = [(C–S)/C] \times 100$$

Where, 'C' is the rate of reduction of free oxalate without any test sample,

'S' is the rate of reduction of free oxalate with a test sample.

3.1.4 Homogenous system of Calcium phosphate (CaP) mineralization

To determine the extent of CaP precipitation, homogenous mineralization system was used to study the extent of in vitro mineral phase formation in the absence of any matrix (Tandon *et al.*, 1998). This *in vitro* homogenous assay system consisted of 0.1 M Tris-Cl Buffer (pH 7.4), 5 mM $CaCl_2$ and 5 mM KH_2PO_4. After incubating this assay system at 37^oC, precipitates obtained were centrifuged and the pellets were resuspended in 0.1 N HCl. The calcium ions (Ca^{2+}) and phosphate ions (HPO_4^{2-}) concentration in the precipitate represented the extent of precipitation (crystallization) of these ions and the sample containing inhibitory biomolecules(s) minimized the extent of their precipitation. The Ca^{2+} and HPO_4^{2-} ions were estimated by the methods as given in sections 3.1.4.1 and 3.1.4.2 respectively (Trinder, 1960; Gomori, 1941).

3.1.4.1 Determination of calcium ions

Principle: Calcium ions get precipitated as naphthylhydroxamate by directly adding slight excess of calcium reagent. After centrifugation, excess of reagent is removed by decantation and unwashed precipitates are dissolved in EDTA. Addition of ferric nitrate results in development of orange color, intensity of which is measured as the amount of calcium ions present in the sample.

Reagents:

- Calcium Reagent – Prepared by mixing two components and the volume raised to 1000 ml by adding distilled water, mixture was filtered and stored in dark reagent bottle. The components were:

  a) 280 mg Naphthylhydroxamic acid in 100 ml (95 ml distilled water + 5 ml ethanolamine + 2 g tartaric acid)

  b) 9 g NaCl in 500 ml distilled water

- Color reagent – 60 g $FeNO_3$ was dissolved in 500 ml acidified distilled water (485 ml distilled water with 15 ml conc. HNO_3). Then, the volume was raised to 1000 ml with distilled water.
- Working Standard – 2 mM $CaCl_2$
- EDTA – 2 g EDTA was dissolved in 1000 ml of 0.1 N NaOH

Procedure:

- 0.1 ml sample was dissolved in 2.5 ml calcium reagent and incubated at room temperature for 30 mins.
- The precipitates were obtained after centrifugation and then dissolved in 1.0 ml of 0.2% EDTA with boiling for 10 mins.
- Finally, 3 ml color reagent was added and the absorbance was measured at 450 nm.

Calculations:

$$\text{Conc. of calcium ions (mM)} = \frac{\text{Absorbance of test}}{\text{Absorbance of standard}} \times \text{Conc. of standard}$$

3.1.4.2 Determination of phosphate ions

Principle: Phosphate reacts with molybdic acid to form phosphomolybdic acid. Treatment of 2-methyl 4-amino sulphate causes reduction of phosphomolybdic acid to form deep blue colored complex which gives absorption maxima at 660 nm

Reagents:

- Molybdic acid – Prepared fresh by mixing 2.5% ammonium molybdate dissolved in distilled water and 10 N H_2SO_4 in the ratio of 10:4.
- Metol Reagent – Prepared by mixing 5% $NaHSO_3$ and 1% metol in distilled water.

- Working Standard – 2 mM Potassium dihydrogen phosphate (KH_2PO_4).

Procedure:

- 0.2 ml sample was added to 1.2 ml molybdic acid and incubated at room temperature for 10 mins. The sample was diluted by 6.8 ml distilled water.
- Then, 0.5 ml metol reagent was added and solutions were vortexed.
- Finally, the mixture was kept at room temperature for 20-30 mins and absorbance was measured at 660 nm.

Calculations:

$$\text{Conc. of phosphate ions (mM)} = \frac{\text{Absorbance of test}}{\text{Absorbance of standard}} \times \text{Conc. of standard}$$

3.1.4.3 Determination of percentage inhibition of calcium phosphate mineralization

The percentage inhibition of CaP mineralization in the presence of the test sample was calculated using following formula:

$$\text{Percentage inhibition} = [(C-T)/C] \times 100$$

Where,

'C' is the concentration of Ca^{2+} or HPO_4^{2-} ions of the precipitate formed in the control system which has distilled water.

'T' is the concentration of Ca^{2+} or HPO_4^{2-} ions of the precipitate formed in the assay system with the test sample.

3.1.5 Homogenous system of growth and demineralization of calcium phosphate mineral phase

The growth and demineralization of the preformed mineral phase consisting of calcium phosphate required initial precipitates of these minerals as obtained by method given in section 3.1.4. In order to study the growth of the preformed mineral

phase, the precipitates formed by the above method were resuspended in the same assay system having calcium and phosphate along with the plant extract. This assay system was incubated at 37^{o}C for 30 mins after which estimation of Ca^{2+} and HPO_4^{2-} ions concentration represented the growth of precipitation of these ions over the previously formed mineral phase.

For demineralization, the preformed mineral phase was resuspended in the assay system with the aqueous extract of *Tribulus terrestris* but without further addition of calcium and phosphate ions. After incubation at 37^{o}C, Ca^{2+} and HPO_4^{2-} ions concentration were estimated in supernatant to determine the demineralization of mineral phase.

The concentration of calcium and phosphate ions of the mineral phase in these test samples was determined. In case of growth of preformed mineral phase, concentration of Ca^{2+} and HPO_4^{2-} ions of the preformed mineral phase was deducted from the final concentration of Ca^{2+} and HPO_4^{2-} ions. The percentage inhibition or simulation caused by different concentrations of the aqueous extract was calculated with respect to control system which has distilled water instead of plant extract. In demineralization of preformed mineral phase, the percentage of Ca^{2+} and HPO_4^{2-} ions demineralised was calculated in the supernatant.

3.1.6 Data Analysis

The data are represented as the mean $\pm$ standard deviation of three replicates.

3.2 Diminution of oxalate induced renal tubular epithelial cell injury by aqueous extract of *Tribulus terrestris*

3.2.1 Cell culture

Normal rat epithelial derived renal tubular epithelial (NRK-52E) cells were obtained from National Centre of Cell Sciences (NCCS, Pune). All chemicals used for the maintenance of the cell lines: Dulbecco's modified Eagles's medium (DMEM), fetal bovine serum (FBS), antibiotics, 0.25% Trypsin-EDTA, Sodium bicarbonate, were tissue culture grade. The cells were maintained as monolayers in Dulbecco's Modified Eagle's Medium (DMEM) with 2.0 mM L-glutamine adjusted to contain 3.7 g/L sodium bicarbonate, 4.5 g/L glucose. Media was supplemented with 1% Penicillin (10,000 units/ml)-Streptomycin (10,000 µg/ml) and 10% FBS. Cells were cultured in 25 cm^2 tissue-culture treated flasks at 37^oC and 5% CO_2 in humidified chambers.

3.2.2 Preparation of Tribulus terrestris extract

A stock solution (20 mg/ml) of the dried aqueous *Tribulus terrestris* extract as obtained in section 3.1.1 was dissolved in DMSO (Dimethyl sulfoxide) [final concentration of the DMSO in the highest concentration of plant extract tested did not exceed 0.4% (v/v) and did not affect the cell proliferation]. 1:100 dilution of the stock was prepared using serum free DMEM and filtered by 0.22 µm syringe filter (Moriyama *et al.*, 2007).

3.2.3 Oxalate-induced cell injury

For experimental purposes, cell were plated at a density of 4 $\times 10^4$ cells/well and cultured to obtain 80% confluence. NRK-52E cells were incubated in DMEM containing 1 mM sodium oxalate in the presence of different concentrations of the

aqueous extract of the test sample (10µg/ml, 25µg/ml and 50µg/ml) for 72 h (Moriyama *et al.*, 2007; Jeong *et al.*, 2005). Cell injury was assessed by measuring the cell viability through trypan blue and monitoring the lactate dehydrogenase (LDH) leakage into the medium.

3.2.4 Cytotoxicity

3.2.4.1 Trypan blue assay

The cytotoxicity of the aqueous extract of *T. terrestris* was assessed by cell viability using trypan blue exclusion method.

Principle: Trypan blue is a diazo dye, which is a vital stain to selectively color dead tissues or cells blue. It is based on the principle that live cells possess intact cell membranes that exclude certain dyes, such as trypan blue, Eosin, or propidium, whereas dead cells do not. Since live cells are excluded from staining, this staining method is also described as a dye exclusion method.

Procedure: For the determination of cell viability, cells were plated at the density of 4 $\times 10^4$ cells/well and cultured to obtain 80% confluence. The medium was replaced with serum-free medium and the cells were treated with various concentrations of the plant extracts (10µg/ml, 25µg/ml and 50µg/ml) for a further 72 h. After the treatment period, cell count (viable and non-viable) was done by mixing 1 part of 0.4% trypan blue and 1 part cell suspension in hemocytometer (Figure 3.1).

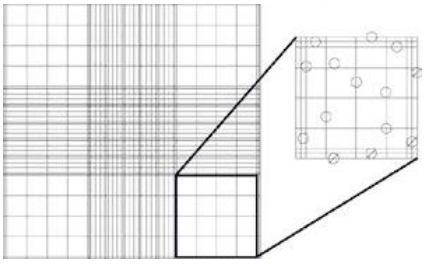


Figure 3.1: Hemocytometer

$$\text{Cell count (cells/ml)} = \frac{\text{total cells counted X dilution factor X } 10^4}{\text{Number of squares observed}}$$

Where, dilution factor = 2,

Number of squares observed = 4

Calculations: The percentage viability for the cells was calculated as follows

Percentage viability = (live cells/total cells) x100

Where, Total cells = Live cells + Dead cells

3.2.4.2 Lactate dehydrogenase (LDH) leakage assay

The cytotoxicity of the aqueous extract of *T. terrestris* was assessed by LDH leakage (Vassault, 1992).

Principle: Lactate dehydrogenase, or LDH, is an enzyme found in the cells of many body tissues, including the heart, liver, kidneys, skeletal muscle and brain. LDH catalyzes the interconversion of pyruvate and lactate with concomitant interconversion of NADH and NAD^+ (Figure 3.2). Cells that have lost membrane integrity release LDH into the surrounding medium, therefore it can be used as a biomarker for cell damage. In the assay, disappearance of NADH is measured at 340 nm with pyruvate as the substrate for enzyme activity.

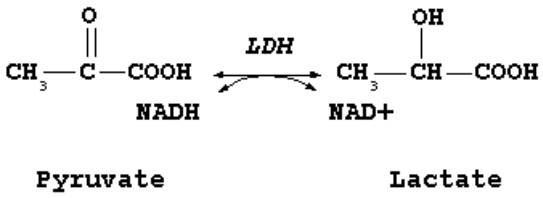


Figure 3.2: Catalytic action of Lactate Dehydrogenase

Procedure: 6.6 mM NADH and 30 mM sodium pyruvate were prepared in Tris-Cl (0.2 M, pH 7.3). Reaction was initiated with the addition of 50 µl of the test sample and the disappearance of NADH was monitored at 340 nm, for 5 min at an interval of 1 min.

Calculations: The percentage of LDH release was calculated as follows:

Percentage of LDH release = (Activity of LDH in the supernatant/ Total LDH activity) X 100 Where, Total LDH activity = Activity of LDH in the supernatant and the LDH activity measured after complete cell lysis achieved by sonication.

3.2.5 Statistical analysis

Data were expressed as mean values of three independent experiments (each in triplicate) and analyzed by the analysis of variance ($p<0.05$) to estimate the differences between values of extracts tested.

3.3 Effect of aqueous extract of *Tribulus terrestris* on experimentally induced nephrolithiatic rats

3.3.1 Materials

Healthy male rats of the Wistar strain weighing 150 – 200 g of equivalent age groups were obtained from the central animal house of Panjab University, Chandigarh, India. The animals were acclimatized for one month in polypropylene cages under hygienic conditions and were provided standard animal feed and water *ad libitum*. All procedures were done in accordance with the ethical guidelines for care and use of laboratory animals and were approved by the local care of the Experimental Animals Committee.

3.3.2 Experimental Groups

All animals were divided into 2 major regimens i.e. prophylactic and curative regimen on the basis of the treatment given for 15 days and 28 days respectively. Each major group was further subdivided into 5 groups having 6 animals each and named as TP1, TP2, TP3, TP4, TP5 and TC1, TC2, TC3, TC4, TC5 respectively. The description of each group with their respective doses and period of treatment is given in Table 3.1. Ethylene glycol induced hyperoxaluria model was used to assess the antilithiatic activity in albino rats (Kaur *et al.*, 2009; Divakar *et al.*, 2010).

The LD_{50} of the aqueous extract of the *Tribulus terrestris* is 19.6g/kg body weight (Al-Ali *et al.*, 2003). The plant extract was suspended in normal saline (Sharififer *et al.*, 2009).

Prophylactic study (15 days)	**Curative study (28 days)**
TP1: Control (2.5ml/kg b.w. normal saline *i.p.* for 15 days)	**TC1:** Control (2.5ml/kg b.w. normal saline *i.p.* for 28 days)
TP2: Hyperoxaluric (0.4% EG + 1% NH_4Cl in drinking water for 15 days)	**TC2:** Hyperoxaluric (1^{st} -15^{th} day: 0.4% EG + 1% NH_4Cl in drinking water; 16^{th} – 28^{th} day: 0.4% EG in drinking water)
TP3: Positive control (0.4% EG + 1% NH_4Cl in drinking water + 750 mg/kg body wt cystone *i.p.* for 15 days)	**TC3:** Positive control (1^{st} -15^{th} day: 0.4% EG + 1% NH_4Cl in drinking water; 16^{th} – 28^{th} day: 0.4% EG in drinking water + 750 mg/kg body wt cystone *i.p.*)
TP4: Test Dose 1 (0.4% EG + 1% NH_4Cl in drinking water + 50 mg/kg body wt *T. terrestris i.p.* for 15 days)	**TC4:** Test Dose 1 (1^{st} -15^{th} day: 0.4% EG + 1% NH_4Cl in drinking water; 16^{th} – 28^{th} day: 0.4% EG in drinking water + 50 mg/kg body wt *T. terrestris i.p.*)
TP5: Test Dose 2 (0.4% EG + 1% NH_4Cl in drinking water + 100 mg/kg body wt *T. terrestris i.p.* for 15 days)	**TC5:** Test Dose 2 (1^{st} -15^{th} day: 0.4% EG + 1% NH_4Cl in drinking water; 16^{th} – 28^{th} day: 0.4% EG in drinking water + 100 mg/kg body wt *T. terrestris i.p.*)

Table 3.1: Animal grouping for *in vivo* studies

3.3.3 Body weight

The animals in various groups were monitored for their physical health and dietary intake. The change in body weight was recorded everyday for animals in all the groups.

3.3.4 Urinary Parameters

3.3.4.1 Urine collection / processing

After the treatment period, the rats were placed in the metabolic cages and urine was collected for 24 h period with 20 µl of 20% sodium azide as a preservative. After measuring urinary volume, the urine was used for the determination of lactate dehydrogenase (LDH) and alkaline phosphatase (ALP). Urinary creatinine was also estimated for the calculation of creatinine clearance.

3.3.4.2 Estimation of lactate dehydrogenase

The activity of Lactate dehydrogenase (LDH) was estimated by the method of Vassault, 1992.

Principle:

$$\underset{\textbf{Pyruvate}}{CH_3CO\,COO^- + NADH + H^+} \xleftrightarrow{LDH} \underset{\textbf{Lactate}}{CH_3.CHOH.COO^- + NAD^+}$$

LDH activity is determined by measuring the rate of disappearance of NADH when pyruvate is converted to lactate. Enzyme activity is measured by estimating the concentration of NADH present in the reaction mixture. NADH absorbs light at 340nm (molar absorbtivity = 6.22 X 10^3 M^{-1} cm^{-1}) whereas NAD^+ does not. The equilibrium for the reaction lies strongly in the direction of reduction of pyruvate and hence, the reaction is followed by measuring the rate of decrease in absorbance of NADH at 340 nm.

Reagents:

- Substrate - 3.5 g K_2HPO_4, 0.45 g KH_2PO_4, 5.35 g NaCl (pH 7.2) and 31 mg of sodium pyruvate were dissolved in 450 ml distilled water
- NADH - 42 mg NADH was dissolved in 4.5 ml 1% $NaHCO_3$

Procedure:

- To a cuvette, 3 ml substrate, 50 µl NADH and 200 µl sample was added.
- The solution was mixed rapidly and a decrease in absorbance was measured at 340 nm.

Calculations:

The activity of Lactate Dehydrogenase was calculated using the following formula:

$$\text{LDH Activity (Units/min/mg protein)} = \frac{\Delta A_{340} / \text{min}}{6.22 \text{ X mg protein/ml sample}}$$

3.3.4.3 Estimation of alkaline phosphatase

The activity of enzyme alkaline phosphatase (ALP) was measured by the method of Bessey *et al.*, 1946.

Principle:

$$p\text{ - nitrophenyl phosphate} + H_2O \xrightarrow[Mg^{2+}]{ALP} p\text{ - nitrophenol} + \text{phosphate}$$

ALP causes the conversion of *p* - nitrophenyl phosphate to *p* – nitrophenol. The rate of formation of the yellow color of *p* – nitrophenol produced by the hydrolysis of *p* - nitrophenyl phosphate in alkaline solution is measured spectrophotometrically at 405nm to calculate the activity of the enzyme.

Reagents:

- ALP Reagent (Reagent 1) – Contains *p* - nitrophenyl phosphate, Mg^{2+} in Tris/Carbonate buffer (pH 10.2)

Procedure:

- To 20 µl sample, add 1.0 ml of Reagent 1.
- Mix well and increase in absorbance was measured at 405 nm with time.

Calculations:

$$\text{ALP Activity (IU/L)} = \frac{(\Delta A_{405} / \text{min}) \text{ X T.V. X } 10^3}{\text{S.V. X Absorbtivity X P}}$$

Where,

T.V. – total reaction volume in µl

S.V. – sample volume in µl

Absorbtivity – 18.8 M^{-1} cm^{-1}

P – cuvette path length – 1 cm

3.3.5 Serum Parameters

3.3.5.1 Preparation of serum

Before sacrificing the rats, the blood was taken from the orbital sinus into a centrifuge tube without anticoagulant and allowed to clot at room temperature and centrifuged at 3000 rpm for 15 mins. The supernatant was collected as serum for the estimation of creatinine and urea.

3.3.5.2 Estimation of creatinine

It is estimated by the Jaffe's method (Bonsnes and Taussky, 1945).

Principle: Creatinine in alkaline solution reacts with picric acid to form an orange red compound. Rate of development of the color is proportional to the concentration of creatinine in the sample and absorbance of the color is measured at 505 nm.

Reagents:

- Picric acid Reagent (Reagent 1) – Picric acid dissolved in distilled water (25.8 mmol/L)
- Sodium Hydroxide Reagent (Reagent 2) – NaOH dissolved in distilled water (95 mmol/L)
- Creatinine Standard – Creatinine dissolved in distilled water (2 mg/dl)

Procedure:

- Mix equal volumes of reagent 1 and reagent 2 and wait for 15 mins before use.
- To 0.1 ml of the sample, add 1.0 ml of the working reagent.
- Mix well and read initial absorbance (A1) at 20 sec after mixing and final absorbance (A2) 80 sec after mixing.
- Appropriate blank and standard were also run in parallel.

Calculations:

$$\Delta A = A2 - A1$$

$$\text{Conc. of creatinine (mg/dl)} = (\Delta A_{test} / \Delta A_{std}) \text{ X Conc. of standard}$$

Where, ΔA_{test} - change in absorbance in presence of test sample

ΔA_{std} - change in absorbance in presence of standard

3.3.5.3 Estimation of urea

Urea in the serum was estimated by GLDH-Urease method (Tiffany *et al.*, 1972)

Principle: The estimation of urea in serum involves the following enzyme catalyzed reactions

$$\text{Urea} + H_2O \xrightarrow{\text{Urease}} 2NH_3 + CO_2$$

$$NH_3 + \alpha\text{ KG} + \text{NADH} \xrightarrow{\text{GLDH}} \text{Glutamate} + NAD^+$$

Where, α KG – α Ketoglutarate

GLDH – Glutamate dehydrogenase

The rate of decrease in absorbance is monitored at 340 nm and is directly proportional to urea concentration in the sample.

Reagents:

- Urea Reagent (Reagent 1) – contains α Ketoglutarate, NADH, Urease, GLDH, ADP in Tris buffer (pH 7.9).
- Urea standard – Urea dissolved in distilled water (50 mg/dl)

Procedure:

- To 20µl of the sample, add 1.0 ml of the Reagent 1.
- Mix well and read initial absorbance (A1) at 20 sec after mixing and final absorbance (A2) 80 sec after mixing.
- Appropriate blank and standard were also run in parallel.

Calculations:

$$\Delta A = A1 - A2$$

$$\text{Conc. of urea (mg/dl)} = (\Delta A_{test} / \Delta A_{std}) \text{ X Conc. of standard}$$

Where, ΔA_{test} - change in absorbance in presence of test sample

ΔA_{std} - change in absorbance in presence of standard

3.3.6 Creatinine Clearance

Creatinine clearance (CrCl) determines the amount of creatinine excreted out of the body per unit time and per unit volume. CrCl was calculated according to standard clearance formula.

$$CrCl\ (ml/min) = (U/S)\ X\ V$$

Where,

U is the urinary concentration of creatinine (mg/dl)

S is the serum concentration of creatinine (mg/dl)

V is the urinary volume in ml/min

3.3.7 Histopathology

After the blood was taken from orbital sinus, the rats were anaesthetized with diethyl ether and sacrificed by decapitation. Immediately after the dissection, perfusion of the internal organs was achieved by normal saline through heart. After perfusion, both kidneys were removed and fixed in formalin.

Procedure:

- Fixation – Small pieces of kidney tissue, after removal of extraneous material were fixed in Bouin. These wre then dehydrated in various grades of ethanol, cleared in benzene and embedded in paraffin wax (M.P. 60 – 62^{o}C). The paraffin sections of appropriate thickness (8 µ) were cut.
- Histopathological Staining [Delafield's Hematoxylin/Eosin (HE)] – First of all, the sections were de-paraffinised in xylene and downgraded through different grades of alcohol to water. These were then stained in D. Hematoxylin for 15-17 mins and then kept under running water. The nuclei of the cells were differentiated in acidic water and ammonia water till these

stained blue. The slides were upgraded in 70% ethanol, dipped in eosin for 1 min, differentiated in 90% alcohol and upgraded to absolute alcohol, cleared in xylene and mounted in DPX. The nuclei, nucleoli and chromatin material stained blue whereas cytoplasm stained pink.

- The slides were viewed under light microscope.

3.3.8 Polarization microscopy of urine from urinary bladder

Immediately after the dissection, urine from the urinary bladder was directly obtained by puncturing with a 5/8 inch needle attached to a 1 ml tuberculin syringe and directly used under polarization microscope to see the presence of crystals in urine. A drop of urine obtained from bladder was spread on a glass slide, covered with a cover slip and visualised under polarized light using Leica DM3000 light microscope.

3.3.9 Statistical Analysis

The data were expressed as mean ± SD and analyzed by ANOVA.

3.4 Purification of antilithiatic protein from *Tribulus terrestris*

The schematic representation used for the purification and characterization of most potent antilithiatic protein is shown in Figure 3.3 (Kaur *et al.*, 2009). The fractions obtained after each step of purification were assayed for their antilithiatic properties by employing a calcium oxalate (CaOx) crystal growth assay (Nakagawa *et al.*, 1985). The protein content was determined by Lowry's method and the extent of purity of the active fractions was determined by SDS-PAGE analysis.

100 g powder of *Tribulus terrestris* extracted with 50mM Tris-Cl buffer (pH 7.4), containing 0.25M NaCl, 1mM PMSF and 0.01% sodium azide for 24hrs with gentle stirring at 4^{o}C

↓

The slurry was centrifuged at 10,000g for 20 min at 4^{o}C to recover the supernatant

↓

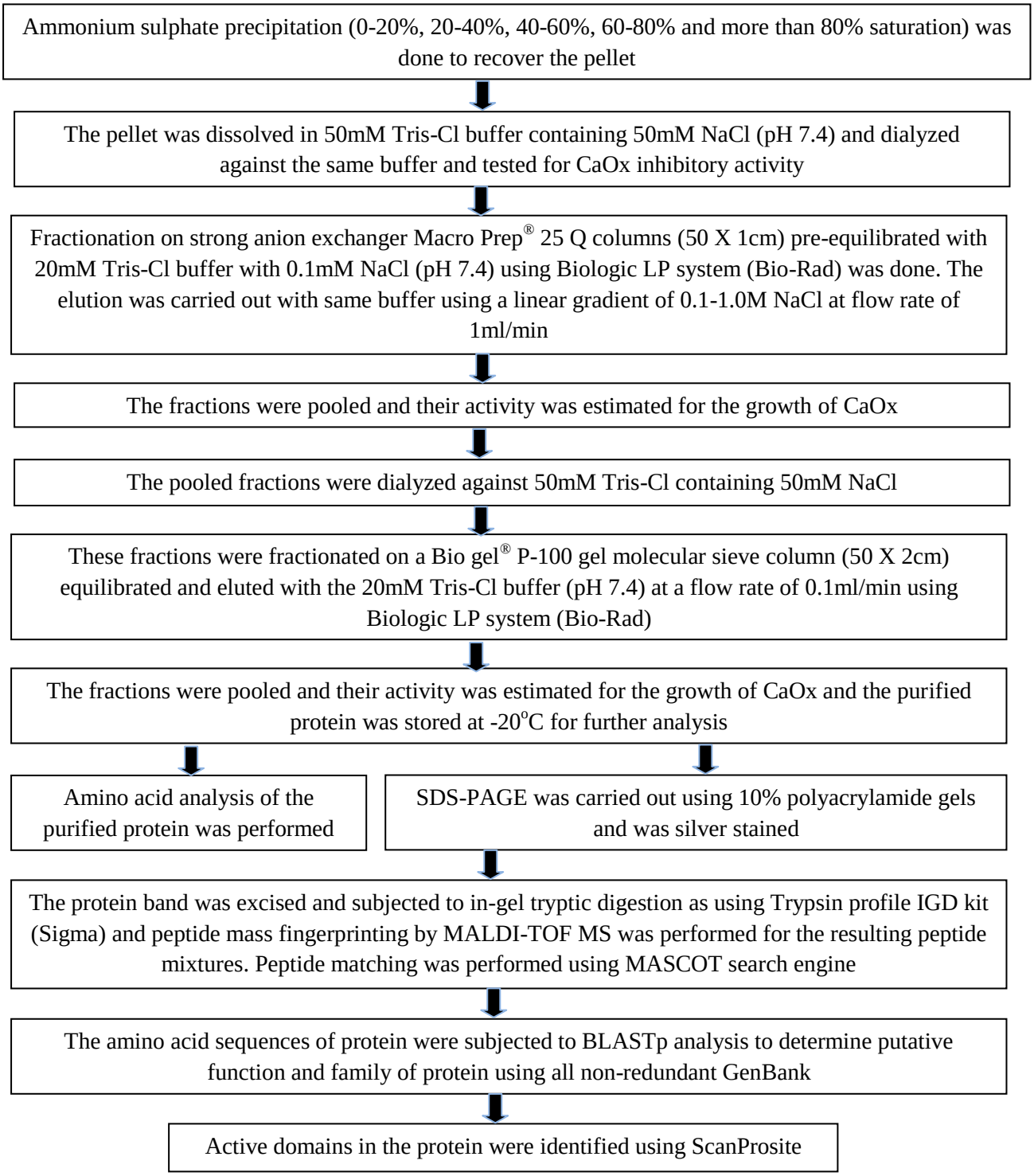


Figure 3.3: Schematic Representation of the purification and characterization of the most potent antilithiatic protein from the fruits of *Tribulus terrestris*

3.4.1 Materials

Materials required were Macro Prep® 25 Q strong anion exchanger (Bio-Rad Laboratories), Bio gel® P-100 gel (Medium, 90 – 180 µm) molecular sieve support (Bio-Rad Laboratories). Solvents used were HPLC grade.

3.4.2 Extraction

Fruits of *Tribulus terrestris* were dried, grinded to obtain a fine powder. To obtain crude protein extract, 100 g of powdered fruits was extracted with the extraction buffer [50 mM Tric-Cl buffer (pH 7.2), containing 0.25 M NaCl, 1mM PMSF and 0.01% sodium azide]. The slurry so formed was stirred continuously for 24 h at 4^{o}C. After 24 h of continuous stirring, the slurry was centrifuged at 10,000 g for 20 min at 4^{o}C. The supernatant was referred to as crude extract of *Tribulus terrestris*.

3.4.3 Ammonium sulphate precipitation

Ammonium sulphate was added to the total volume of the homogenate to obtain a precipitate formed between 0 - 20%, 20 - 40%, 40 - 60%, 60 - 80% and more than 80% saturation. The precipitates obtained were dialyzed against 50 mM Tris-Cl Buffer (pH 7.4) containing 50 mM NaCl. The dialyzed samples were checked for CaOx inhibitory activity as described in section 3.1.3. After obtaining the sample having highest inhibitory activity, it was stored at -20^{o}C and used for further purification of antilithiatic protein.

3.4.4 Ion exchange chromatography

The active dialyzate obtained from ammonium sulphate precipitation was centrifuged at 10,000 rpm for 15 min at 4^{o}C to remove insoluble material and then filtered by Whatman filter paper. The Macro Prep® 25 Q strong anion exchanger, after removal of ethanol, was pre-equilibrated with 20 mM Tris-Cl buffer containing 0.1 M NaCl at

pH 7.4. The column (50 X 1cm) was packed with pre-equilibrated strong anion exchanger slurry and washed with same buffer by two bed volumes of anion exchanger. Protein sample was loaded in injecting loop and the column was eluted with buffer A and B [Buffer A - 20 mM Tris-Cl buffer with 0.1 mM NaCl (pH 7.4); Buffer B - 20 mM Tris-Cl buffer with 1 mM NaCl (pH 7.4)]. The column was eluted by a linear gradient of 0.1-1.0M NaCl at flow rate of 1ml/min using Biologic LP system (Bio-Rad) using the method as shown in Table 3.2.

A total of 160 fractions (1 ml) were collected. The absorbance at 280 nm for each fraction was read and the elution profile was made using LP Data view software version 1.03. The fractions under each peak were pooled and tested for their inhibitory potency and active fraction was dialyzed against buffer A to remove excess salt.

Time (minutes)	Flow rate (ml/min)	Buffer
0 – 20	1.0	Buffer A
20 – 90	1.0	0 – 90% gradient of Buffer A to Buffer B
90 – 130	1.0	Buffer B
130 – 160	1.0	Buffer A

Table 3.2: Method used for Anion exchange chromatography

3.4.5 Molecular sieve chromatography

The molecular sieve resin [Bio gel® P-100 (Medium, 90 – 180 µm), Bio-Rad Laboratories] was equilibrated with 20 mM Tris-Cl buffer (pH 7.4). The most active fraction that was dialysed after anion exchange chromatography was loaded on molecular sieve chromatography (50 X 2 cm) and eluted with the same buffer using

Biologic LP system at a flow rate of 0.1 ml/min. Fractions of 1 ml were collected throughout the elution. The absorbance at 280 nm for each fraction was read and the elution profile was made using LP Data view software version 1.03. The potent fraction with CaOx inhibitory activity was freeze dried.

3.4.6 SDS-PAGE

Electrophoresis of the active fraction obtained after ammonium sulphate precipitation, anion exchange and molecular sieve chromatography was carried on 10% gel under denaturizing conditions in the presence of reducing agent. Electrophoresis separation was accomplished in a Bio-Rad Mini Protean gel system at 100 V for an average 1.5h. Gels were stained with silver nitrate.

3.4.7 Quantification of proteins

The Lowry's method was used for the quantitative estimation of proteins (Lowry *et al.*, 1951).

Principle: The phenolic group of tyrosine and tryptophan residues in a protein produces a blue-purple color complex with Folin-Ciocalteau reagent which has sodium tungstate molybdate and phosphate. Thus, the intensity of the color depends on the amount of aromatic amino acids present and the absorption is measured at 750 nm.

Reagents:

- BSA stock solution (1 mg/ml)
- Lowry's Reagent: It was prepared by mixing 98 ml component (a) and 2 ml component (b)

  (a) 50ml 2% Na_2CO_3 mixed with 50 ml 0.1 N NaOH solution

  (b) 10 ml 1% $CuSO_4$ mixed with 10 ml 2% Sodium Potassium Tartarate

- Folin-Ciocalteau Reagent (1 N): Prepared fresh by diluting the commercial reagent with water in 1:1 ratio.

Procedure:

- Added 0.9 ml water to 0.1 ml test sample.
- 5.0 ml Lowry's reagent was added to it mixed well and allowed to stand at room temperature for 10 mins.
- Finally, 0.5 ml Folin-Ciocalteau reagent was added. The solution was mixed again and incubated at 37^{o}C for 30 mins. Similarly, standard and blank tubes were run in parallel and then absorbance was taken at 750 nm.

Calculations:

$$\text{Conc. of protein (mg/ml)} = \frac{\text{Absorbance of test}}{\text{Absorbance of standard}} \times \text{Conc. of Standard}$$

3.5 Characterization of purified antilithiatic protein

3.5.1 Materials

Trypsin profile IGD kit (Sigma, St. Lois, USA) was used for protein trypsinization. HPLC column used was Pico Tag (Waters). Solvents used were HPLC grade. MASCOT, Scan Prosite and BLASTp were online tools used.

3.5.2 Total amino acid composition

Total amino acid content in the protein was estimated after acid hydrolysis of the purified protein sample by the method of Elkin and Wasynczuk, 1987. For hydrolysis, 200 µl of HCl/Phenol solution was added and the sample was dried in nitrogen gas prior to its hydrolysis at 105^{o}C in oven for 24 h. This causes release of free amino acids from the protein. Derivatization of free amino acids after hydrolysis was performed by phenylisothiocyanate (PITC), ethanol, water and triethylamine in ratio

1:7:1:1. The derivarized sample was loaded on silica based Pico Tag (Waters; 3.9 mm X 15 cm) column. Before loading, the sample was diluted with a solution made up of disodium hydrogen phosphate with 5% acetonitrile. About 20 µl of this solution was loaded on the column, thus indicating that about 1µg of the protein is loaded. Elution was done under high pressure using a gradient of sodium acetate trihydrate in 6% acetonitrile and 60% acetonitrile. The detection was done at 254 nm and the temperature was kept at 46^{o}C during the separation procedure.

Eluent A: Sodium acetate trihydrate in 6% acetonitrile

Eluent B: 60% acetonitrile in water

3.5.3 Peptide mass fingerprinting

Figure 3.4: MALDI-TOF MS

Peptide mass fingerprinting was done using Matrix-Assisted Laser Desorption/Ionization –Time of Flight Mass Spectrometry (Figure 3.4). It is a very sensitive technique and can determine the molecular mass of a protein even in a pico

mole amount. In Peptide mass fingerprinting, protein is digested with trypsin and various small peptides thus obtained are subjected to MALDI-TOF MS which determines the molecular weight of each peptide of the trypsinized protein.

The protein band obtained after purification was excised and subjected to in-gel tryptic digestion by using Trypsin profile IGD kit (Sigma). The resulting peptide mixtures were eluted on the sample plate with the matrix solution (10 mg/ml α-cyano-carboxycinnamic acid in 50% acetonitrile/0.1% trifloroacetic acid). Matrix and samples were deposited on the MALDI probe and allowed to air dry. The spectrum was then recorded on Bruker Ultraflex MALDI-TOF MS operating at 20,000 V with the nitrogen laser focussed at 337 nm.

3.5.4 Peptide Matching

Peptide matching was performed using MASCOT search engine (http://www.matrixscience.com) assuming that the peptides were monoisotopic, carbamidomethylated at cysteine residues and oxidised at methionine residues. A mass tolerance was 1.2 Da and only 1 maximal cleavage was allowed for peptide matching. Probability-based MOWSE (Molecular Weight Search) score was calculated using the formula [-10 log (P)], where P was the probability that the observed match was a random event.

3.5.5 Putative function and Domain Prediction

The amino acid sequences of protein were subjected to BLASTp analysis to determine putative function and family of protein using all non-redundant GenBank. The presence of the active domains in the random hit was found using the online tool, ScanProsite. The amino acid sequence of the hit was used as an input and the presence of the domains were searched.

3.6 Protective effect of potent protein biomolecules of *Tribulus terrestris* on NRK-52E

The purified protein sample obtained after molecular sieve chromatography as described in section 3.4.5 was dialyzed (Molecular weight cut off 3kDa) and then reconstituted in 0.22µ filtered autoclaved distilled water. Cytotoxicity was assessed as discussed earlier (Section 3.2.3 and 3.2.4) with test sample concentration as 1µg/ml, 2µg/ml and 4µg/ml. A positive control, cystone, was also tested for its protective potency towards oxalate injured renal epithelial cells (NRK-52E).

RESULTS

4.1 Antiurolithiatic potency of *Tribulus terrestris in vitro*

A physico-chemical theory of urolithiasis considers urine as a supersaturated solution in which homogenous or heterogeneous nucleation can lead to initial crystal formation, which can then aggregate and grow. Calcium oxalate (CaOx) is found to be the predominant component of most of the kidney stones accounting for more than 80% stones.

4.1.1 Effect of Tribulus terrestris aqueous extract on the nucleation of calcium oxalate (CaOx)

Figure 4.1 elucidates the effect of different concentrations of the aqueous extract of *Tribulus terrestris*. Nucleation of CaOx crystals is a predominant step in the initiation of the kidney stones. As depicted from the figure, saturation was seen in the inhibitory potency towards nucleation with increase in concentration. With respect to control (with no plant sample), the percentage inhibition remained constant at 71.4% despite increase in the concentration of *Tribulus terrestris* extract to the tune of 25µg/ml and 100µg/ml but a minor decrease was observed in the inhibition for 50µg/ml as 71.1%. As the concentration of *Tribulus terrestris* extract was further increased to 200µg/ml, the percentage inhibition increased to 100% but was reduced to 85.7% for 400µg/ml. The percentage inhibition was restored to 100% with 1000µg/ml of the extract.

4.1.2 Effect of Tribulus terrestris aqueous extract on the growth of calcium oxalate (CaOx)

Literature reveals that there is an aggregation of the super saturated ions in the urine on the nidus formed through nucleation resulting in the growth of kidney stones. Rate of deposition of oxalate ions were measured at λ214nm in a heterogeneous assay system. Percentage inhibition of deposition of oxalate ions was calculated for the test

sample with respect to control (with no plant sample). *Tribulus terrestris* extract showed inhibition towards CaOx crystal growth in a concentration dependent manner, as shown in Figure 4.2. The percentage inhibition with 25µg/ml of plant sample was found to be 17.6%. As the concentration increased to 50µg/ml, 100µg/ml and 200µg/ml, the inhibition was almost constant in the range of 65-70% but inhibition increased significantly with 400µg/ml and 1000µg/ml to 95.3% and 99.7% respectively.

4.1.3 Effect of Tribulus terrestris aqueous extract on inhibition of precipitation of calcium and phosphate, growth and demineralization of the preformed mineral phase using the homogenous system of in vitro mineral phase formation

Calcium phosphates are the second most commonly found kidney stones worldwide. Kidney stones can be treated at initiation, growth and demineralization of the preformed mineral phase. A homogenous assay system was used to evaluate the inhibitory potency of different concentrations of the aqueous extract of *Tribulus terrestris* on all the 3 phases *in vitro*. Concentration of calcium and phosphate ions was measured in the precipitates for initial mineralization and growth on preformed mineral phase while, supernatants were used to estimate Ca^{2+} and $HPO4^{2-}$ release by the action of the plant. Percentage inhibition was calculated with respect to the control (with no plant sample).

During initial mineralization, aqueous extract of *Tribulus terrestris* showed a small amount of inhibition with respect to calcium ions with all test sample concentrations from 25µg/ml to 1000µg/ml. The extract didn't prove to be a good inhibitor of phosphate ions during initial mineral phase formation.

The aqueous extract of the plant was not found to be a potent inhibitor of growth of preformed crystallization. With respect to calcium ions, the plant extract was able to inhibit their concentration in comparison to 25µg/ml and 50µg/ml, though its activity was lost on increasing the concentration. A small amount of inhibition of phosphate ions (8.8%) was observed at 50µg/ml plant extract. This potency was also lost on increasing the test sample concentrations.

The ability to demineralise preformed mineral phase by *Tribulus terrestris* extracts was tested by estimating the concentration of calcium and phosphate ions released from the precipitate in the supernatant. It is evident from Figure 4.3 that the aqueous extract of *Tribulus terrestris* proved to be very effective in demineralization of the preformed mineral phase in comparison to initial mineralization and growth of preformed mineralization. The percentage of calcium and phosphate ions released on treatment with various concentrations of plant extracts is quite remarkable. The percentage release of calcium ions was 52% with 1000µg/ml of the aqueous extract which was able to demineralise 62% phosphate ions in comparison to the control.

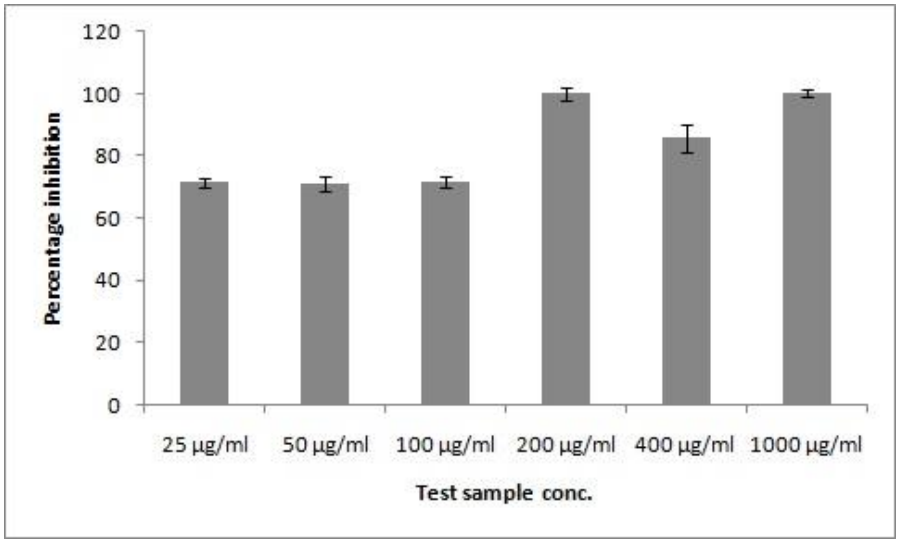


Figure 4.1: Effect of aqueous extract of *Tribulus terrestris* on CaOx crystal nucleation. Results are expressed as Mean ± S.D. of 3 different experiments.

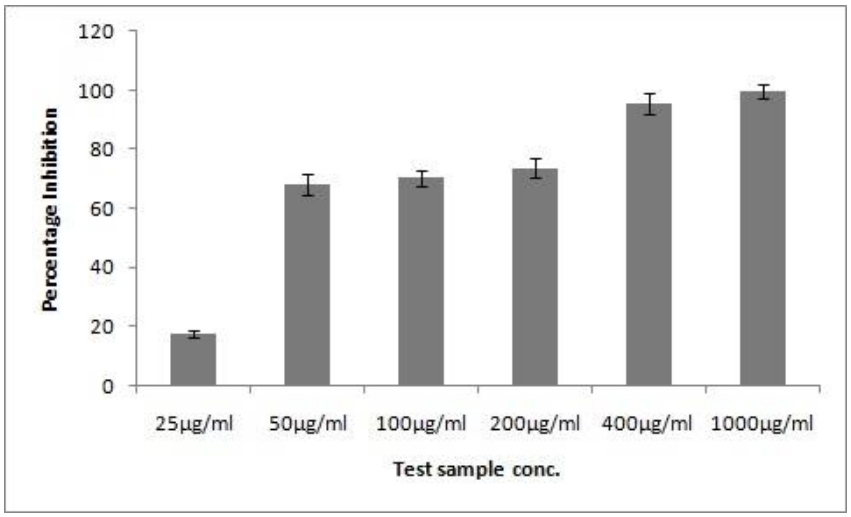
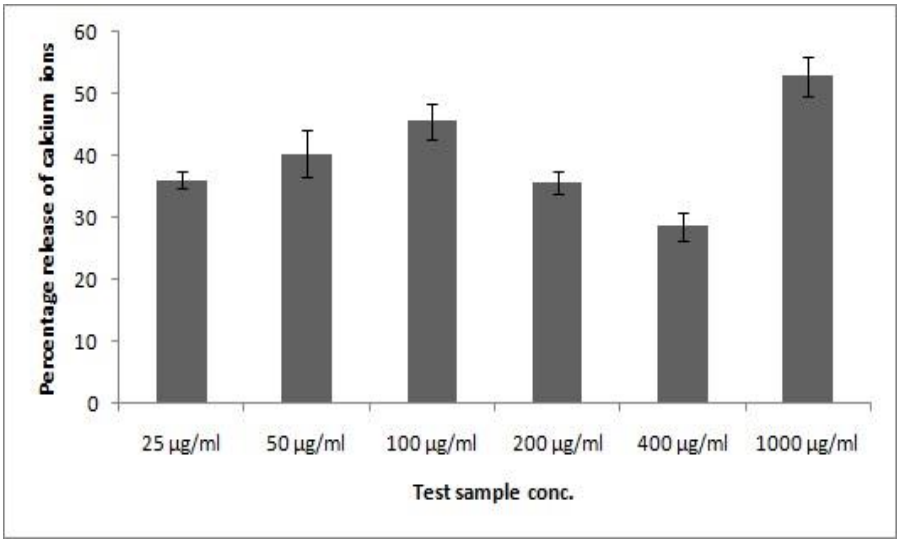


Figure 4.2: Effect of aqueous extract of *Tribulus terrestris* on CaOx crystal growth. Results are expressed as Mean ± S.D. of 3 different experiments.



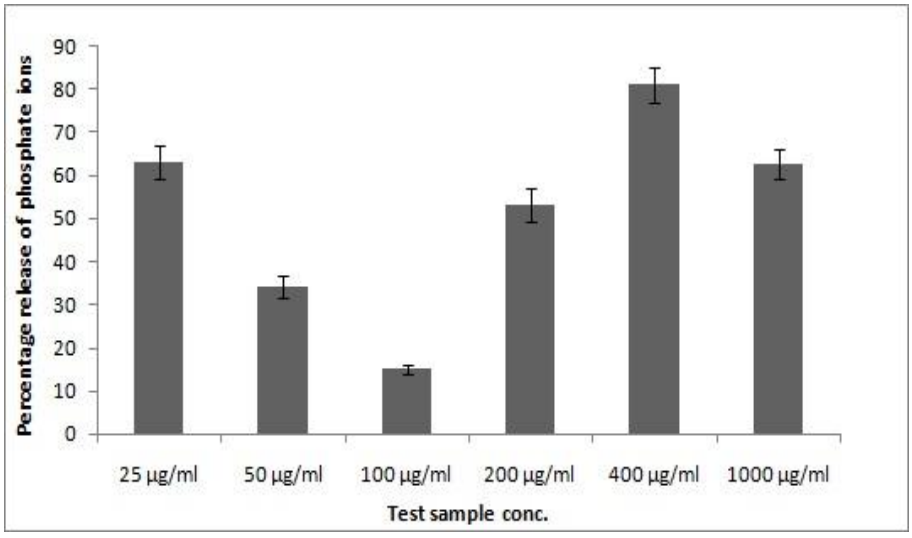


Figure 4.3: Percentage of calcium (Ca^{2+}) and phosphate (HPO_4^{2-}) ions released by *Tribulus terrestris* on demineralization of preformed mineral phase of CaP. Results are expressed as Mean ± S.D. of 3 different experiments.

4.2 Diminution of oxalate induced renal tubular epithelial cell injury by aqueous extract of *Tribulus terrestris*

4.2.1 Cell Viability

Figure 4.4 depicts the protective effect of the aqueous extract of *Tribulus terrestris* towards the renal tubular epithelial cells, NRK-52E.The oxalate induced a significant

injury to the cells which could be ascertained by a decrease in viability from 100% in the controls (untreated cells) to 73.9%. However, the injury due to oxalate was significantly reduced in those cells treated with the *Tribulus terrestris* extracts. As the concentration of the extract increased from 10µg/ml to 50µg/ml, the percentage viability improved showing that the plant is showing a protective effect towards the oxalate caused injury to the renal cells in a concentration dependent manner. The plant extract alone (50µg/ml, containing 0.4% DMSO) had no effect on the cell injury in the absence of oxalate indicating that even at the highest concentration of DMSO used there was no cytotoxicity to the cells . The percentage viability with 10µg/ml, 25µg/ml and 50µg/ml was 81.6 $\pm$ 6.9, 84.9 $\pm$ 1.9 and 89.1 $\pm$ 6.9 respectively.

4.2.2 Cell Injury

Lactate dehydrogenase (LDH) is a stable cytosolic enzyme that is released when the cell is lysed or there is any injury on the cell membrane. A significant increase in LDH release was seen when the NRK-52E cells were exposed to oxalate alone, as observed in Figure 4.5. When NRK-52E cells were treated with the plant extract at varying concentrations(10µg/ml, 25µg/ml and 50µg/ml) along with oxalate (1mM) for 72h, a reduction in oxalate-induced cell injury was observed as assessed by a decreased LDH release. Again, it was seen that the plant extract alone had no significant effect on the measures of cell injury in the absence of oxalate. The percentage LDH release for 10µg/ml, 25µg/ml and 50µg/ml was observed to be 126.5 $\pm$ 4.2, 112.6 $\pm$ 5.2 and 109.8 $\pm$ 1.0 respectively after treatment with oxalate and the plant extract with respect to control.

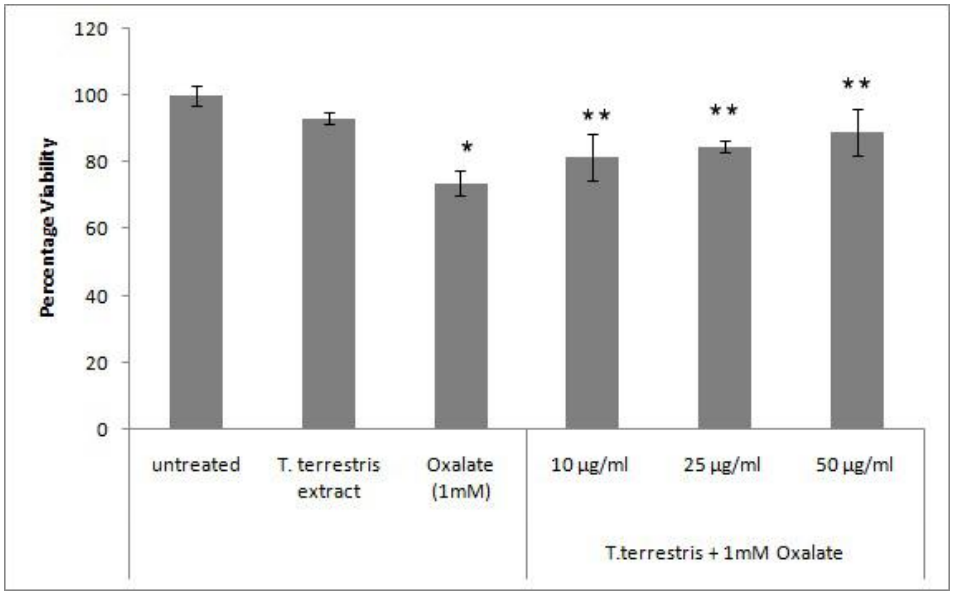


Figure 4.4: Effect of *Tribulus terrestris* on the percentage viability of NRK-52E. Data are mean ± SEM of three independent observations. *$p<0.05$ *versus* untreated control. **$p<0.05$ *versus* oxalate control.

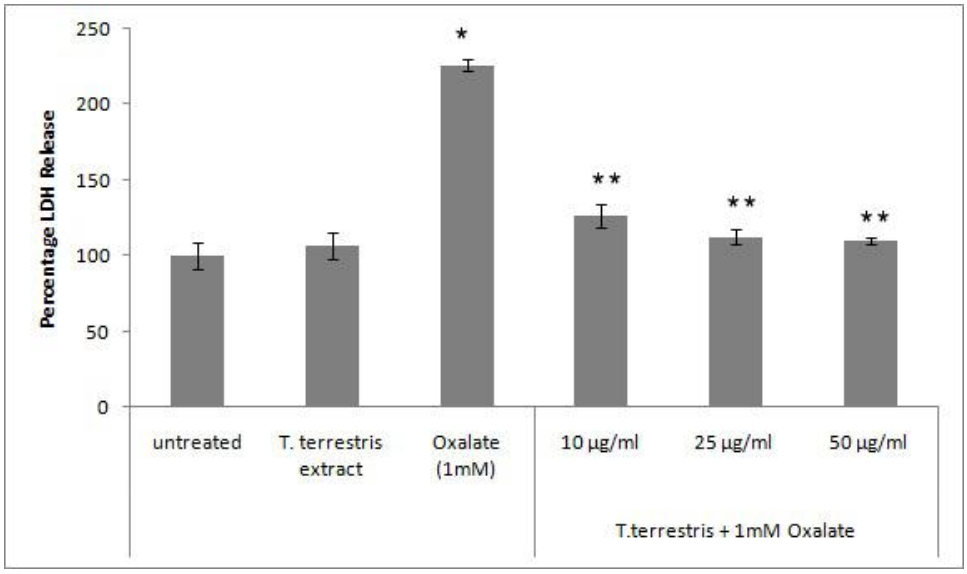


Figure 4.5: Effect of *Tribulus terrestris* on the percentage LDH release of NRK-52E. Data are mean ± SEM of three independent observations. *$p<0.05$ *versus* untreated control. **$p<0.05$ *versus* oxalate control.

4.3 Effect of aqueous extract of *Tribulus terrestris* on experimentally induced nephrolithiatic rats

To elucidate the effect of aqueous extract of *Tribulus terrestris in vivo*, which proved to be a potent inhibitor of CaOx crystal nucleation and growth, CaP crystallisation and protective towards oxalate induced renal epithelial cell injury in NRK-52E, an ethylene glycol induced hyperoxaluric model was used as described in section 3.4. The LD_{50} of *Tribulus terrestris* was found to be 19.6 g/kg body wt from the literature. Hence the test doses were decided to be 50 mg/kg body wt and 100 mg/kg body wt. The rats were examined daily for their general health and physical activity. The dose of EG +NH_4Cl and aqueous extract was well tolerable and rats progressed well during the course of treatment period.

4.3.1 Prophylactic regimen

As described in section 3.3.2, the treatment period was of 15 days which included the administration of EG + NH_4Cl in the drinking water and different doses of the aqueous extract of *Tribulus terrestris i.p.* from day 1 to day 15. Following parameters were studied to assess the efficacy of *Tribulus terrestris* in preventing urolithiasis.

4.3.1.1 Body weight

After treatment with ethylene glycol and ammonium chloride for 15 days, a significant ($p<0.01$) decrease in body weight of group TP2 animals was observed. A decrease of 37% was observed in comparison to control animals (group TP1). Figure 4.6 shows the body weights of all the animals after the treatment period. Group TP3 animals were given 750 mg/kg body wt cystone (positive control) which showed a mere 8% decrease in body weights. As depicted from the figure, the administration of 50 mg/kg body wt (TP4) and 100 mg/kg body wt (TP5) plant extracts exhibited lesser

decrease in body weights (31% and 17% respectively w.r.t. control group TP1, *p<0.01*).

4.3.1.2 Urinary Analysis

4.3.1.2.1 Lactate Dehydrogenase

Lactate dehydrogenase (LDH), a cytosolic enzyme, released on cell injury, is a biomarker for kidney injury. As depicted in Figure 4.7, activity of urinary LDH were highly elevated in hyperoxaluric group TP2 to the tune of 310% (*p<0.01*). Group TP3 animals, which were dosed to Cystone (positive control), showed a significant (*p<0.01*) restoration in the activity of LDH (161% increase was observed w.r.t. TP1 group animals). However, with the treatment of aqueous extract of *Tribulus terrestris* to group TP4 and TP5, activity of LDH was decreased in a dose dependent pattern in comparison to the hyperoxaluric group TP2 leading to an increase of 295% and 266% as compared to TP1 group animals.

4.3.1.2.2 Alkaline Phosphatase

Renal calculi is known to cause damage to renal epithelium causing the release of enzymes like alkaline phosphatase (ALP), which is a potent biomarker. Activity of urinary ALP was measured in all treatment groups as shown in Figure 4.8. The treatment of ethylene glycol and ammonium chloride for 15 days in drinking water to group TP2 animals (urolithiatic animals) has increased the activity of ALP by about 229% as compared to control group TP1 animals significatly (*p<0.01*). With the dosing of cystone to group TP3 animals, activity of ALP was decreased significantly as compared to urolithiatic group (*p<0.01*), exhibiting an increase of 59% in comparison to group TP1 animals. Administration of the plant extract showed its protective effect in a dose dependent manner as the activity of ALP was found to be

decreased in group TP4 and TP5 w.r.t. urolithiatic group TP2 animals significantly ($p<0.05$). The percentage increase observed in the activity of ALP in group TP4 and TP5 in comparison to TP1 animals was 210% and 188% respectively.

4.3.1.3 Serum Analysis

4.3.1.3.1 Urea

Serum urea content was estimated in the serum isolated from the blood of all animals from retro-orbital sinus after the treatment period of 15 days. From the Figure 4.9, it can be seen that in group TP2 animals, urea content in serum increased by 105% in comparison to group TP1 (control) animals. Administration of cystone, along with EG and NH_4Cl, showed a little increase of 21% w.r.t. group TP1 animals. On the other hand, treatment with plant extract, serum urea content of groups TP4 and TP5 was found to be significantly ($p<0.05$) lower in comparison to group TP2. An increase of 75% and 60% was observed on treatment with aqueous extract of *T. terrestris* to group TP4 and TP5 animals respectively w.r.t. control TP1 animals.

4.3.1.3.2 Creatinine

The creatinine content estimated in the serum after 15 days exposure of ethylene glycol and ammonium chloride showed an increase of almost 108% in group TP2 as compared to control group TP1 as seen in Figure 4.10. Administration of cystone along with induction of urolithiatic conditions to group TP3 animals restored the levels of serum creatinine to normalcy significantly in comparison to group TP2 ($p<0.05$) as seen by a mere increase of 10% w.r.t. TP1 group animals. Although, both groups TP4 and TP5 showed a significant ($p<0.05$) increase (61% and 45% respectively) in the creatinine content as compared to control group TP1 animals, but

still the serum creatinine content of the animals was significantly less with respect to urolithiatic group TP2 animals.

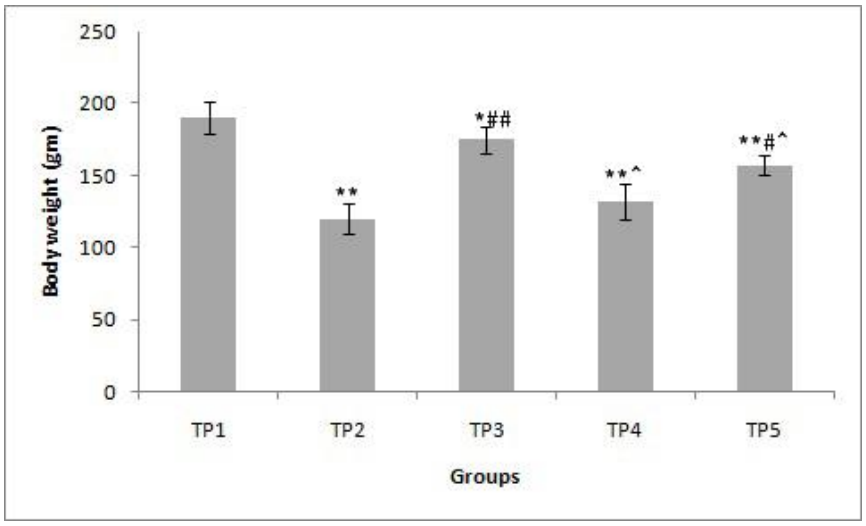


Figure 4.6: Effect on body weights after the treatment period of 15 days. $^{*}p<0.05$, $^{**}p<0.01$: Indicate significant change in comparison to control group TP1. $^{\#}p<0.05$, $^{\#\#}p<0.01$: Indicate significant change in comparison to hyperoxaluric group TP2. $^{\wedge}p<0.05$,$^{\wedge\wedge}p<0.01$: Indicate significant change in comparison to positive control group TP3.

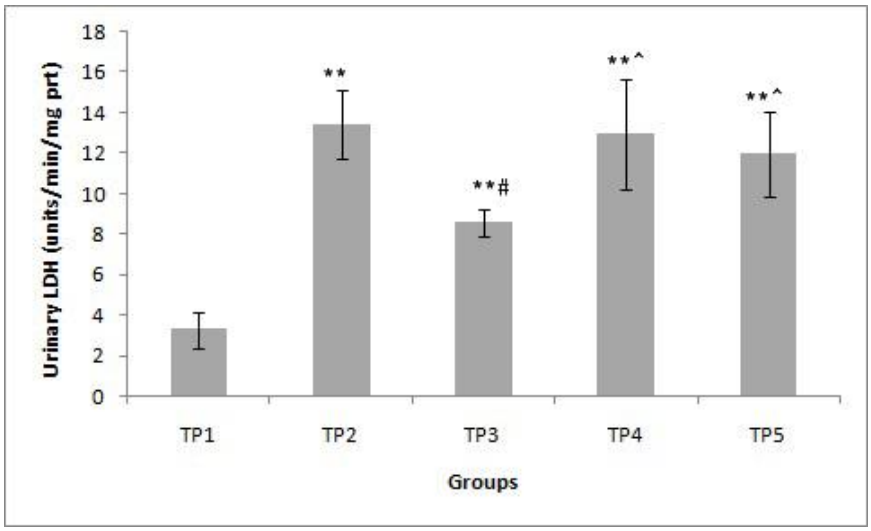


Figure 4.7: Activity of Lactate Dehydrogenase after the treatment period of 15 days. $^{*}p<0.05$, $^{**}p<0.01$: Indicate significant change in comparison to control group TP1. $^{\#}p<0.05$, $^{\#\#}p<0.01$: Indicate significant change in comparison to hyperoxaluric group TP2. $^{\wedge}p<0.05$,$^{\wedge\wedge}p<0.01$: Indicate significant change in comparison to positive control group TP3.

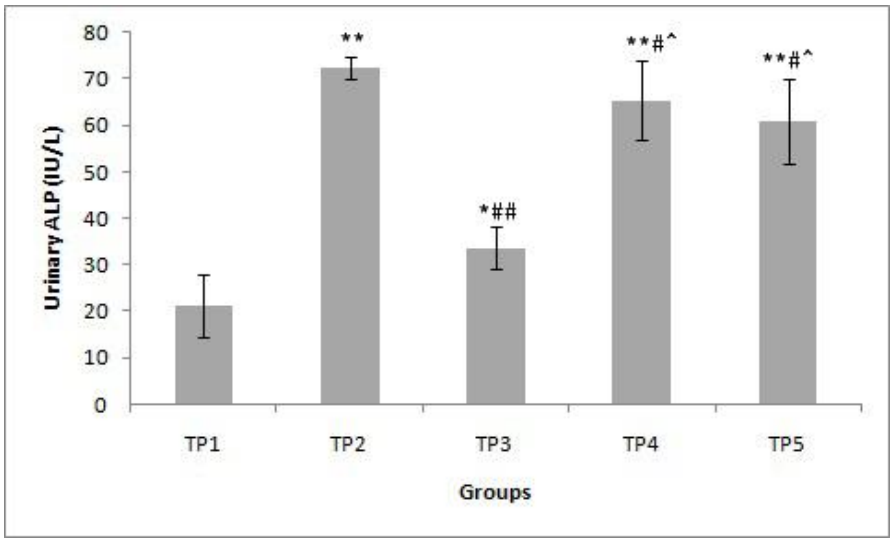


Figure 4.8: Activity of Alkaline Phosphatase after the treatment period of 15 days. $^{*}p<0.05$, $^{**}p<0.01$: Indicate significant change in comparison to control group TP1. $^{\#}p<0.05$, $^{\#\#}p<0.01$: Indicate significant change in comparison to hyperoxaluric group TP2. $^{\wedge}p<0.05$,$^{\wedge\wedge}p<0.01$: Indicate significant change in comparison to positive control group TP3.

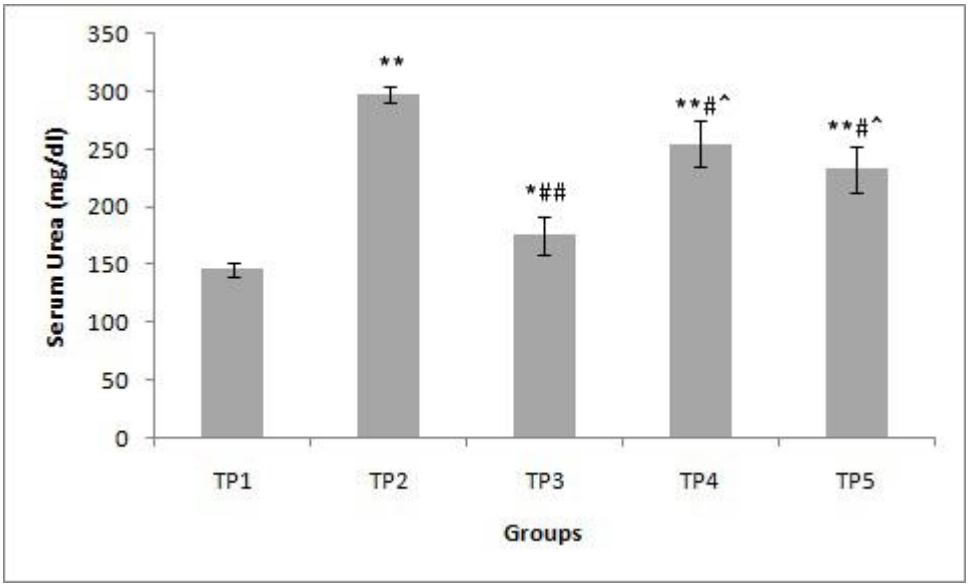


Figure 4.9: Content of Serum Urea after the treatment period of 15 days. $^{*}p<0.05$, $^{**}p<0.01$: Indicate significant change in comparison to control group TP1. $^{\#}p<0.05$, $^{\#\#}p<0.01$: Indicate significant change in comparison to hyperoxaluric group TP2. $^{\wedge}p<0.05$,$^{\wedge\wedge}p<0.01$: Indicate significant change in comparison to positive control group TP3.

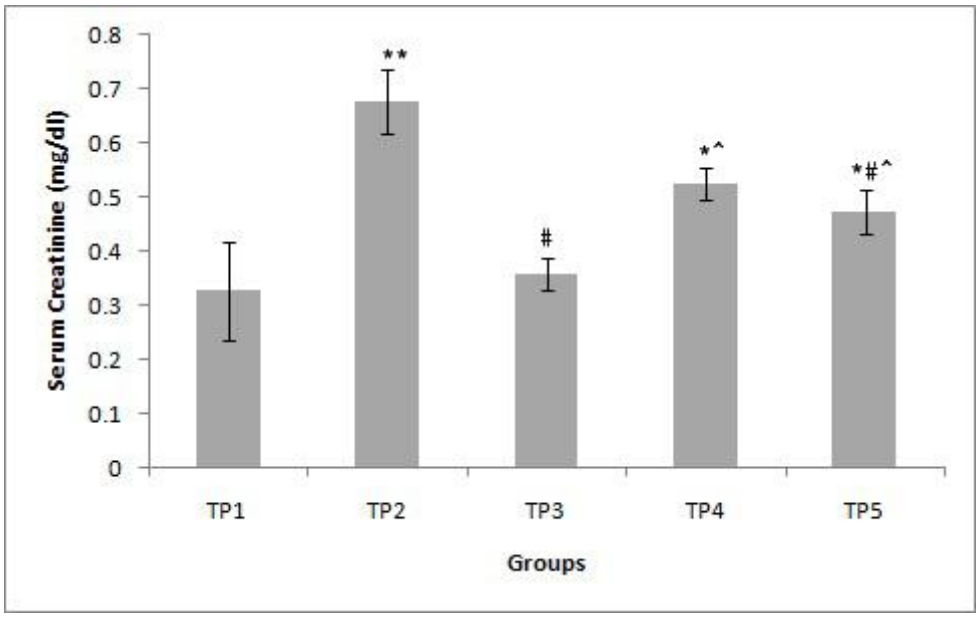


Figure 4.10: Content of Serum Creatinine after the treatment period of 15 days. $^{*}p<0.05$, $^{**}p<0.01$: Indicate significant change in comparison to control group TP1. $^{\#}p<0.05$, $^{\#\#}p<0.01$: Indicate significant change in comparison to hyperoxaluric group TP2. $^{\wedge}p<0.05$,$^{\wedge\wedge}p<0.01$: Indicate significant change in comparison to positive control group TP3.

4.3.1.4 Creatinine Clearance

Creatinine clearance was calculated by the formula as given in section 3.3.6 using serum and urine creatinine content and is graphically represented in Figure 4.11 for a treatment period of 15 days. Figure shows that after 15 days of exposure of ethylene glycol and ammonium chloride, the animals in group TP2 showed a marked decrease in creatinine clearance (60%) as compared to control animals (Group TP1). Simultaneous administration of cystone along with the exposure of EG + NH_4Cl restored the levels to normal with a decrease of 11% in comparison to group TP1. 50 mg/kg body wt and 100 mg/kg body wt plant doses prove to be effective in increasing the creatinine clearance rate in comparison to group TP2 animals, though there was a

significant decrease in both group TP4 and TP5 in comparison to group TP1 animals (55% and 43% respectively, $p<0.01$).

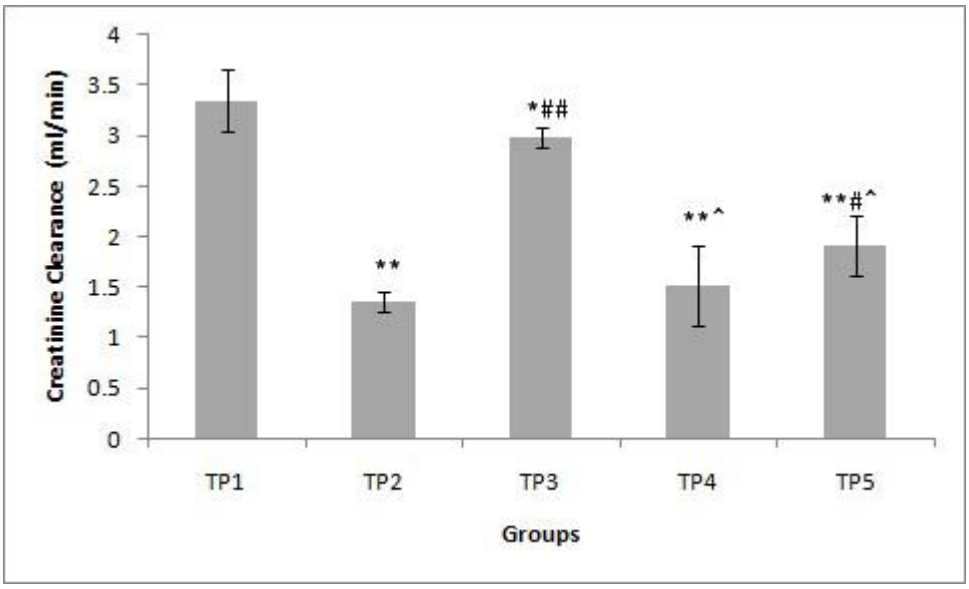


Figure 4.11: Effect on Creatinine Clearance after the treatment period of 15 days. $^{*}p<0.05$, $^{**}p<0.01$: Indicate significant change in comparison to control group TP1. $^{\#}p<0.05$, $^{\#\#}p<0.01$: Indicate significant change in comparison to hyperoxaluric group TP2. $^{\wedge}p<0.05$, $^{\wedge\wedge}p<0.01$: Indicate significant change in comparison to positive control group TP3.

4.3.1.5 Histopathological Analysis of renal tissue

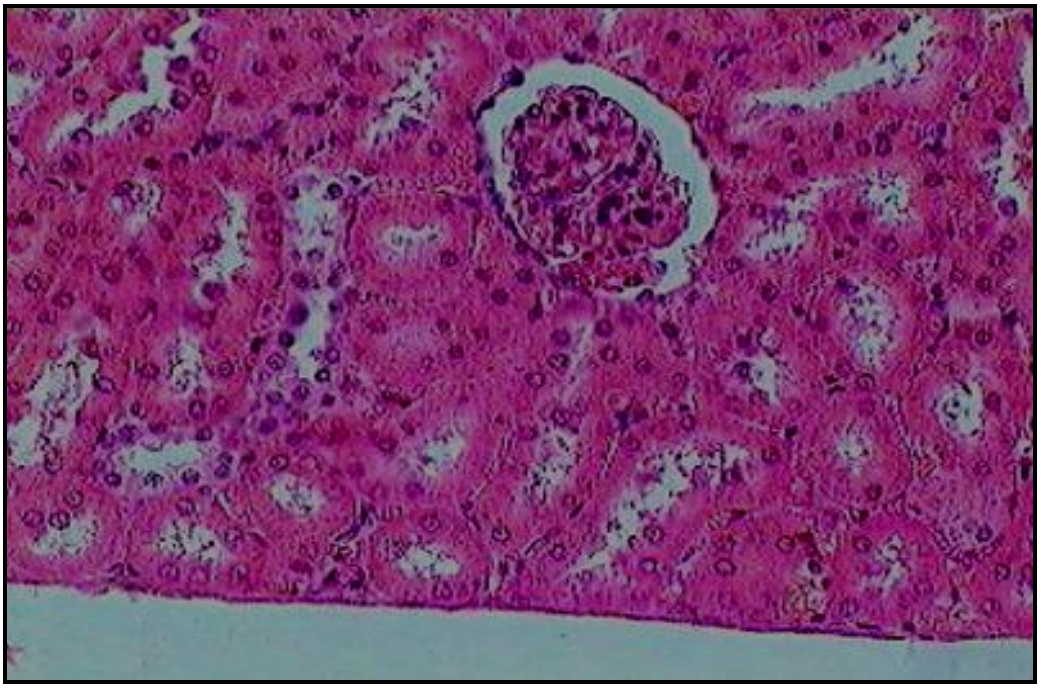
The histopathological examination of kidney also supported the serum and urine biochemistry results as described in Figure 4.12. Control rats showed normal glomerular and tubular histology (TP1), whereas, in all the stone forming rats (group TP2), severe damage was observed as compared to the normal rat kidney architecture in form of glomerular and peritubular damage. The proximal tubules appeared focally ectasic. Flattened epithelium with focal vacuolar degeneration and single cell necrosis bordered the tubules. Irregular crystals were present inside the tubules. Tubular epithelial cells show degenerative changes, including cytoplasmic swelling, cytoplasmic vacuolar degeneration, and atypical cell membrane disruption.

Concurrent treatment with the Cystone and extract was found to reduce such changes in kidney histology induced by EG and NH_4Cl. In group TP3 animals, Cystone was able to restore changes to normalcy to a greater extent. On the other hand, administration of the plant sample at different doses was also able to restore the morphology as compared to the urolithiatic group TP2 animals. The signs of inflammation are reduced in a dose dependent manner. There was reduced crystal deposition in group TP4 and TP5 animals.

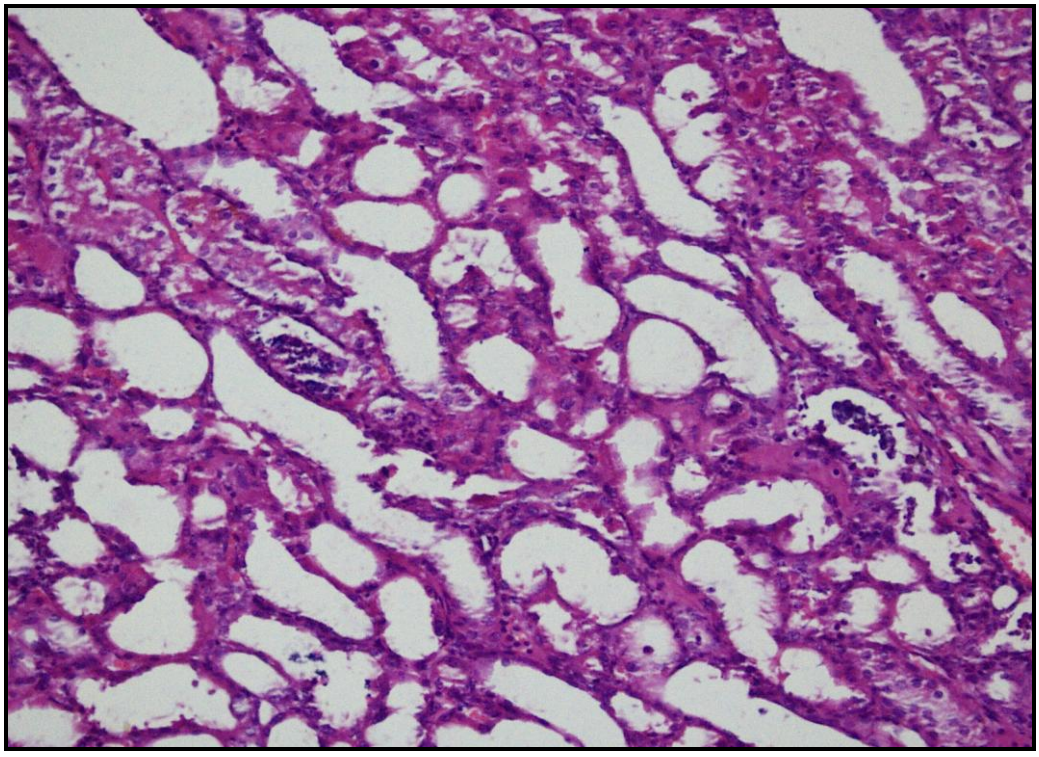
Table 4.1 illustrates the various histopathological features of the kidneys of rats of different treatment groups. Grades are based on observation of renal damage as visualized under light microscopic: "-" Not detected; "+" Mild; "++" Moderate; "+++" Severe.

Histopathological Feature	**Control (TP1)**	**Hyperoxaluric (TP2)**	**Cystone treated (TP3)**	**Dose 1 of *T. terrestris* (TP4)**	**Dose 2 of *T. terrestris* (TP5)**
Glomerular congestion	-	+++	+	+++	++
Epithelial desquamations	-	+++	-	+++	++
Vascular dilation	-	+++	-	++	+
Necrosis	-	++	+	+	+

Table 4.1: Effect on renal histopathology in control and experimental animals after the treatment period of 15 days



Group TP1



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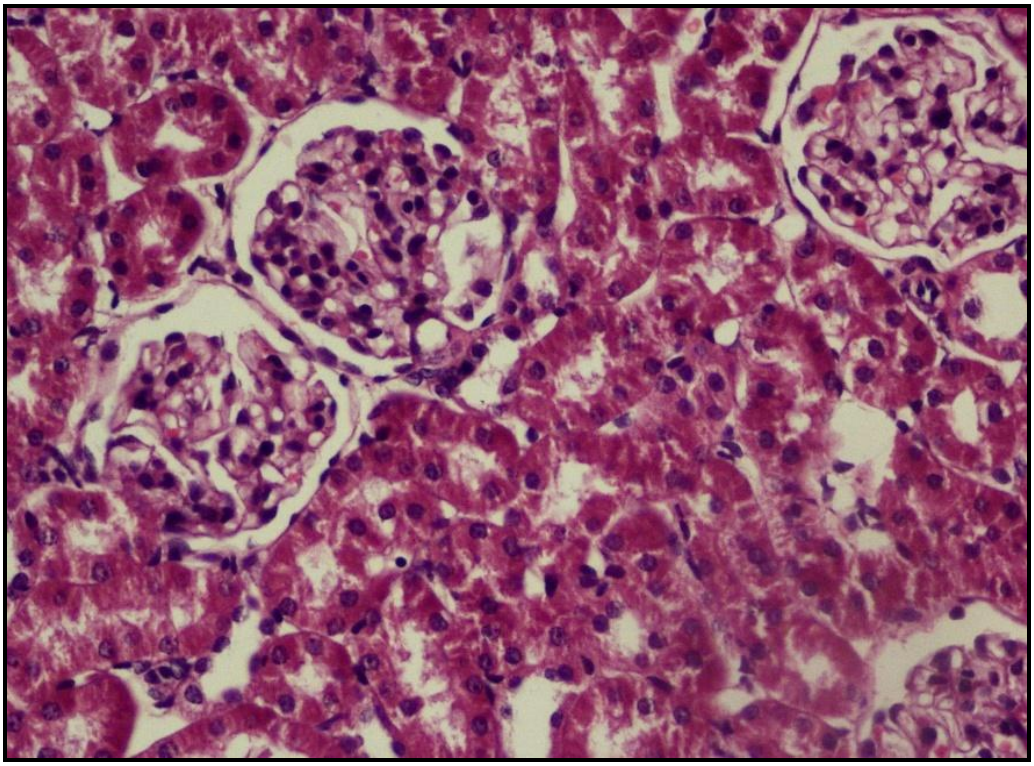

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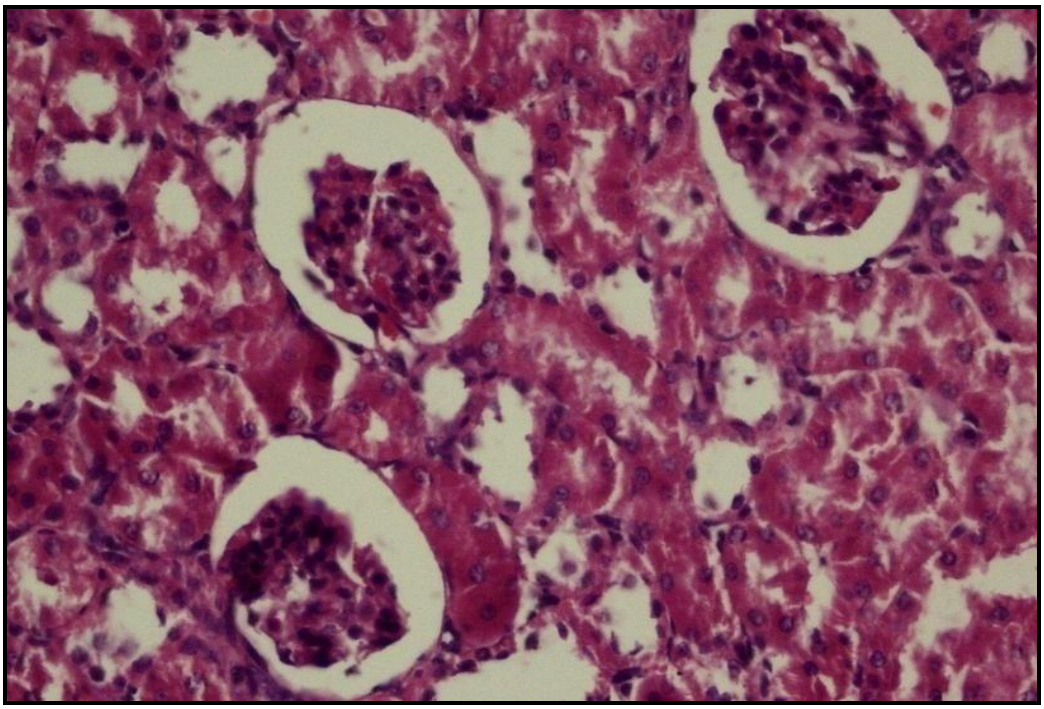
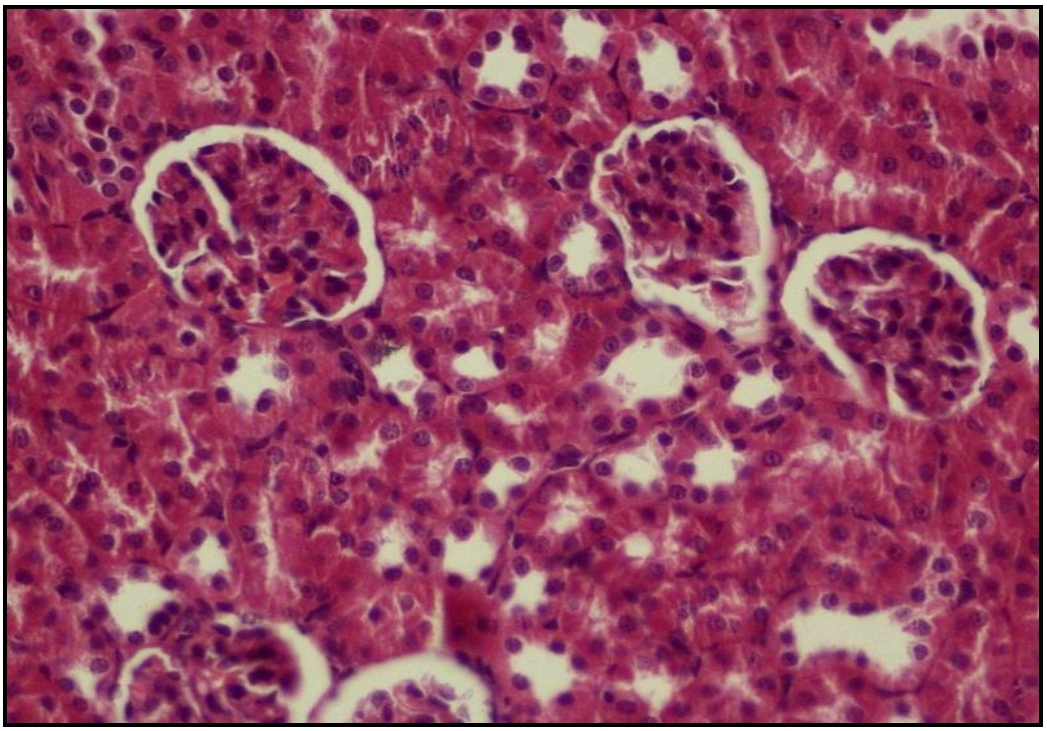


Group TP4



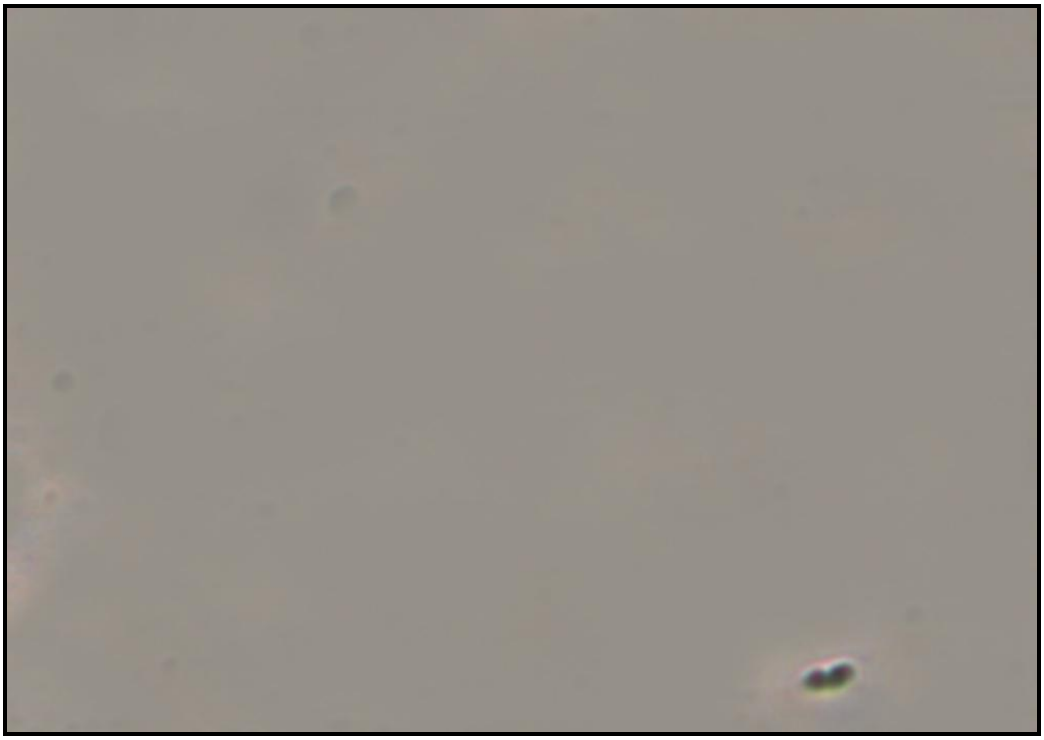
Group TP5

Figure 4.12: Renal histopathology of rats under light microscope given treatment for 15 days. Magnification: 400X. Group TP1: Untreated, Group TP2: Hyperoxaluric, Group TP3: Cystone treated, 750 mg/kg body wt, Group TP4: Dose 1 of *Tribulus terrestris*, 50 mg/kg body wt, Group TP5: Dose 2 of *Tribulus terrestris*, 100 mg/kg body wt

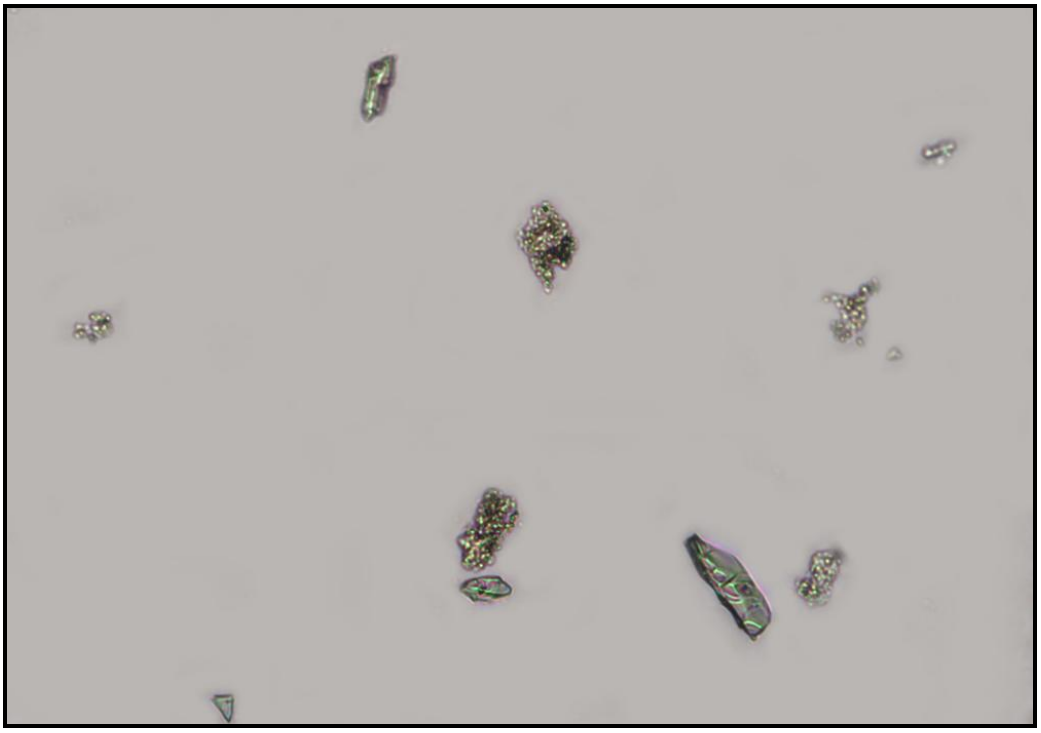
4.3.1.6 Polarization Microscopy of Urine

On microscopic examination after examination period of 15 days, the urine of the control group (TP1) was found to be devoid of crystals or any similar structures (Figure 4.13). In calculi induced rats, the urine samples showed abundant, large crystals with the characteristic shape of CaOx monohydrate, CaOx dihydrate and triple phosphate (TP2). Phosphate stones may add to the chances of retention of CaOx crystals. Aggregates were also visible in group TP2 animals exposed to EG + NH_4Cl treatment.

Cystone, a standard antilithiatic marketed drug, significantly decreased the number of crystals in prophylactic regimen (TP3).The analysis of crystalluria showed that untreated rats excreted more CaOx of both types, and as larger crystals, than excreted by rats treated by Cystone or plant extract. The urine of rats of group TP4 and TP5, which were administered with 50 mg/kg body wt and 100 mg/kg body wt plant extract, exhibited a property of breaking down the crystals to smaller fragments thus facilitating their faster elimination from the body.



Group TP1



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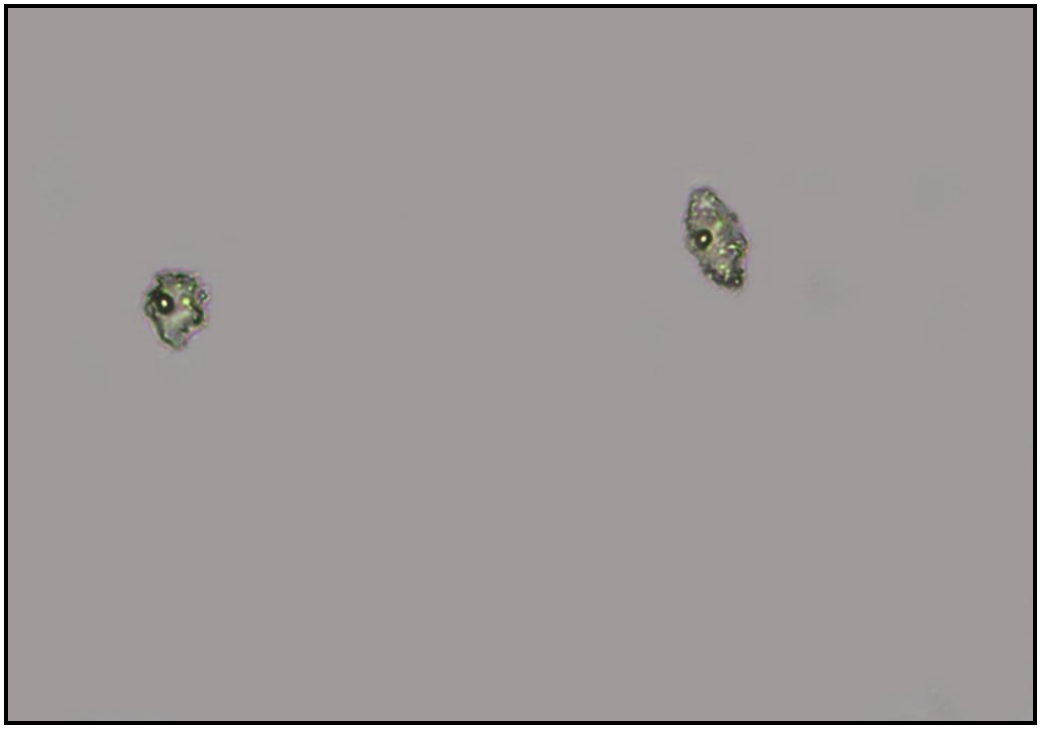
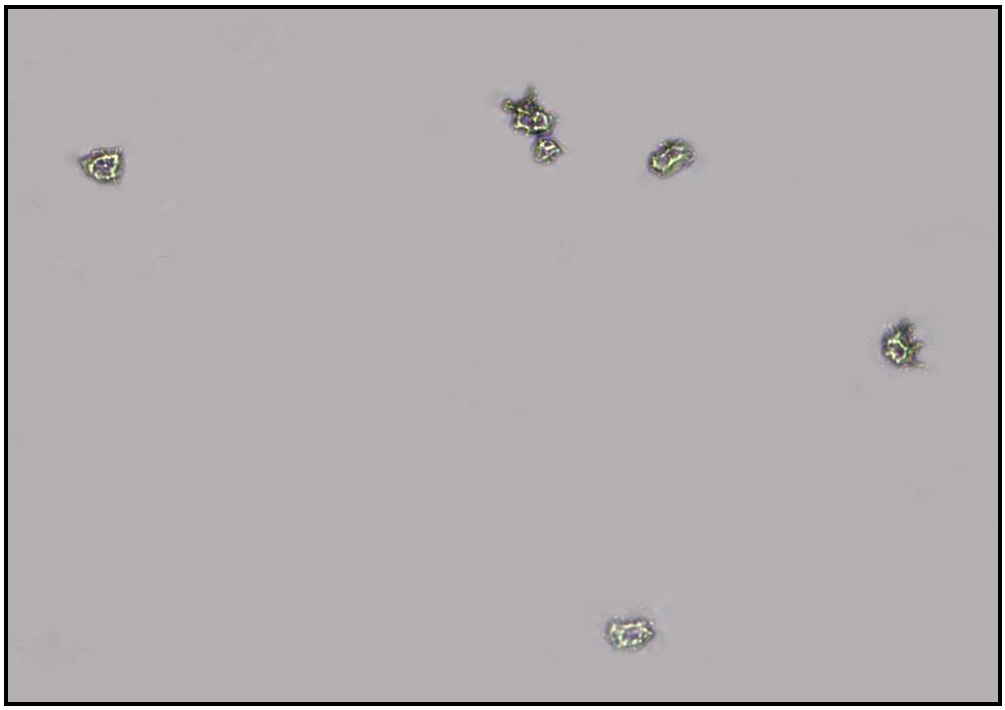
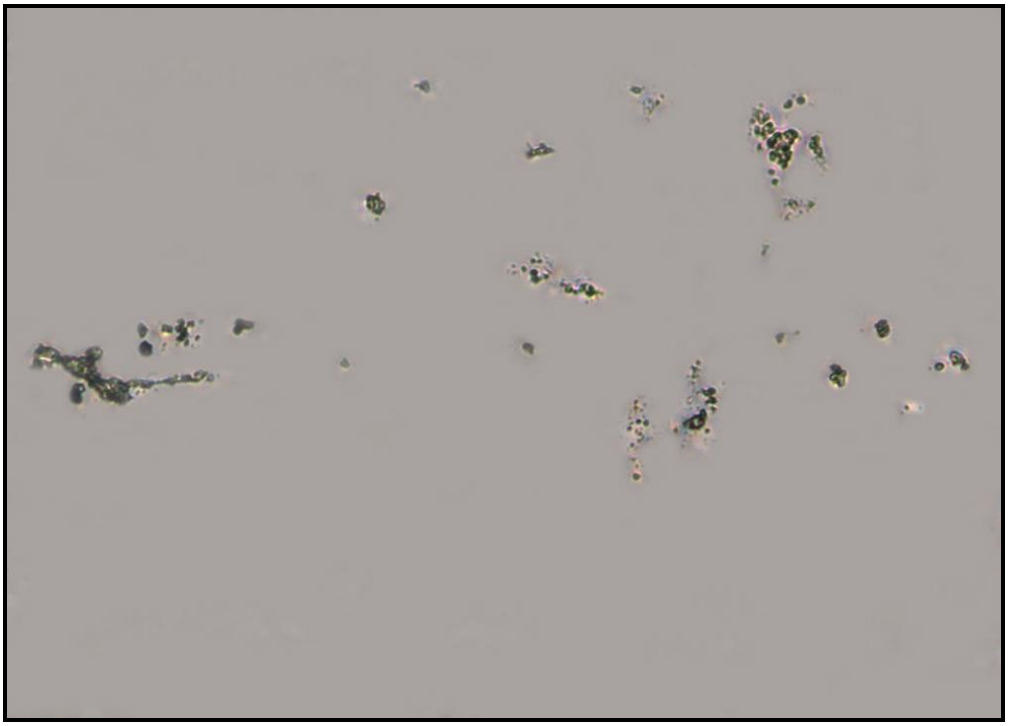


Group TP3



Group TP4



Group TP5

Figure 4.13: Polarization Micrographs of rat's urine given treatment for 15 days. Magnification: 100X. Group TP1: Untreated, Group TP2: Hyperoxaluric, Group TP3: Cystone treated, 750 mg/kg body wt, Group TP4: Dose 1 of *Tribulus terrestris*, 50 mg/kg body wt, Group TP5: Dose 2 of *Tribulus terrestris*, 100 mg/kg body wt.

4.3.2 Curative regimen

In the study for the curative effect, nephrocalcinosis was induced in the kidneys of rats in both untreated and treated groups with EG (0.4%) and NH_4Cl (1%) treatment for 15 days, following the plan as given in section 3.3.2. Thereafter, NH_4Cl treatment was withdrawn and treatment with vehicle and the test material was respectively started to the untreated and the treated groups for another period of 13 days along with EG treatment.

Various urinary and serum parameters were tested along with renal histopathology and polarization microscopy of rat's urine.

4.3.2.1 Body weight

Exposure to ethylene glycol and ammonium chloride lead to a significant ($p<0.01$) decrease in body weight of group TC2 animals to the tune of 46% in comparison to control group TC1. This decrease was even more than the decrease observed in prophylactic treatment for the corresponding group. Figure 4.14 shows the body weights of all the animals after the treatment period. Group TC3 animals dosed with 750 mg/kg body wt cystone (positive control), showed a 15% decrease in body weights w.r.t. TC1 group animals, though it significantly cured the lithogenic effect as seen in TC2 group ($p<0.01$). Both the doses of the aqueous extract of *Tribulus terrestris* upon administration (group TC4 and TC5), restored the decrease by lithogenic treatment in comparison to group TC2 significantly ($p<0.05$) with a decrease of 28% and 25% w.r.t. TC1 group animals respectively.

4.3.2.2 Urinary Analysis

4.3.2.2.1 Lactate Dehydrogenase

Lactate dehydrogenase (LDH), a cytosolic enzyme, is a kidney injury biomarker. Group TC2 animals which were exposed to ethylene glycol and ammonium chloride, showed highly elevated activity of urinary LDH, as depicted in Figure 4.15 to the levels of 445%, significant ($p<0.01$) as compared to group TC1 animals. Activity of LDH in group TC2 was found to be quite high than the corresponding group in prophylactic regimen, TP2. Cystone treated animals (Group TC3) showed the decrease in the activity of urinary LDH significantly ($p<0.01$) as compared to urolithiatic group TC2 exhibiting an increase of 91% in comparison to TC1 control group animals. However, with the treatment of aqueous extract of *Tribulus terrestris* to group TC4 and TC5, activity of LDH was decreased in a dose dependent pattern in comparison to the hyperoxaluric group TC2, though activity was still high as compared to control group TC1 animals ($p<0.01$), showing an increase of 294% and 253% respectively.

4.3.2.2.2 Alkaline Phosphatase

Activity of alkaline phosphatase (ALP), another kidney injury biomarker, was measured in all treatment groups as shown in Figure 4.16. The treatment of ethylene glycol and ammonium chloride in drinking water to group TC2 animals (urolithiatic animals) has increased the activity of ALP by about 252% as compared to control group TC1 animals ($p<0.01$). On treatment of cystone to group TC3 animals, activity of ALP was decreased significantly as compared to urolithiatic group ($p<0.01$) and was quite comparable to control group animals TC1 (52% increase). Administration of the aqueous extract of *Tribulus terrestris* in different doses showed its effect in a

dose dependent manner as the activity of ALP was found to be decreased in group TC4 and TC5 w.r.t. urolithiatic group TC2 animals, though increase was 172% and 156% as compared to group TC1 animals respectively.

4.3.2.3 Serum Analysis

4.3.2.3.1 Urea

Serum urea content was estimated in the serum isolated from the blood of animals of all treatment groups from retro-orbital sinus after the treatment period of 28 days. From Figure 4.17, serum urea content was found to be increased by 125% in group TC2 animals, quite significant ($p<0.01$) as compared to control group TC1 animals. Treatment with cystone as a curative agent to group TC3 animals, exhibited a 34% increase as compared to control group TC1. On the other hand, treatment with plant extract, serum urea content of groups TC4 and TC5 was found to be lower in comparison to group TC2 animals significantly ($p<0.01$), with an increase of 63% and 56% respectively w.r.t. control TC1 group animals. *Tribulus terrestris* extract was effective in decreasing urea content to normalcy in curative regimen as increase w.r.t. control was less in comparison to being a preventive agent (75% and 60% for TP4 and TP5 respectively).

4.3.2.3.2 Creatinine

The creatinine content estimated in the serum after exposure of ethylene glycol and ammonium chloride showed an increase of almost 164% in group TC2 as compared to control group TC1 which was quite significant ($p<0.01$), as seen in Figure 4.18. Administration of cystone along lithogenic treatment to group TC3 animals restored the levels of serum creatinine to normalcy significantly in comparison to group TC2 ($p<0.01$), with a small increase of 5% in comparison to control group animals TC1.

Although, *Tribulus terrestris* treated groups TC4 and TC5 showed a significant ($p<0.01$) increase (121% and 102% respectively) in the creatinine content as compared to control group TC1 animals, but still the serum creatinine content of the animals was significantly ($p<0.05$) less with respect to urolithiatic group TC2 animals.

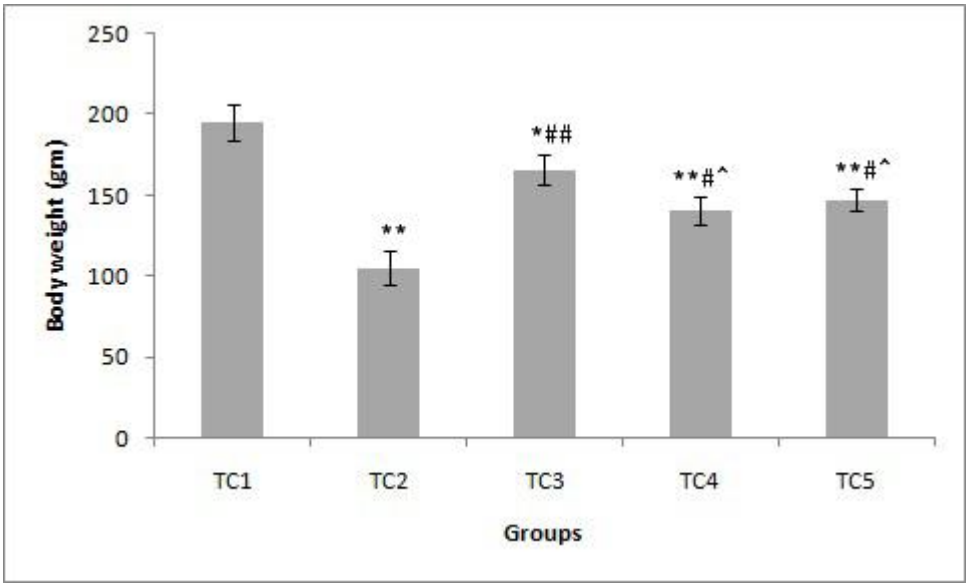


Figure 4.14: Effect on body weights after the treatment period of 28 days. $^{*}p<0.05$, $^{**}p<0.01$: Indicate significant change in comparison to control group TC1. $^{\#}p<0.05$, $^{\#\#}p<0.01$: Indicate significant change in comparison to hyperoxaluric group TC2. $^{\wedge}p<0.05$, $^{\wedge\wedge}p<0.01$: Indicate significant change in comparison to positive control group TC3.

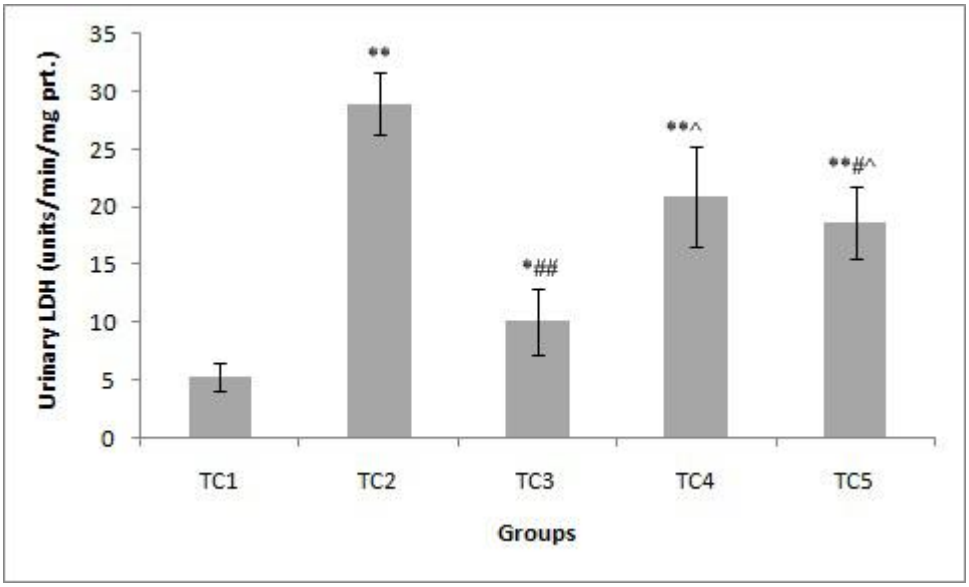


Figure 4.15: Activity of Lactate Dehydrogenase after the treatment period of 28 days. $^{*}p<0.05$, $^{**}p<0.01$: Indicate significant change in comparison to control group TC1. $^{\#}p<0.05$, $^{\#\#}p<0.01$: Indicate significant change in comparison to hyperoxaluric group TC2. $^{\wedge}p<0.05$, $^{\wedge\wedge}p<0.01$: Indicate significant change in comparison to positive control group TC3.

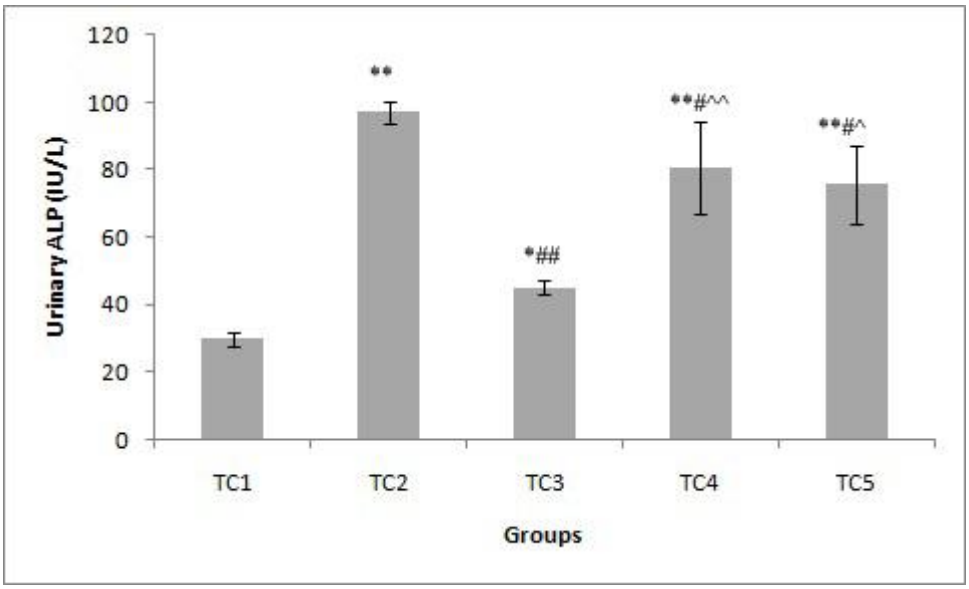


Figure 4.16: Activity of Alkaline Phosphatase after the treatment period of 28 days. $^{*}p<0.05$, $^{**}p<0.01$: Indicate significant change in comparison to control group TC1. $^{\#}p<0.05$, $^{\#\#}p<0.01$: Indicate significant change in comparison to hyperoxaluric group TC2. $^{\wedge}p<0.05$, $^{\wedge\wedge}p<0.01$: Indicate significant change in comparison to positive control group TC3.

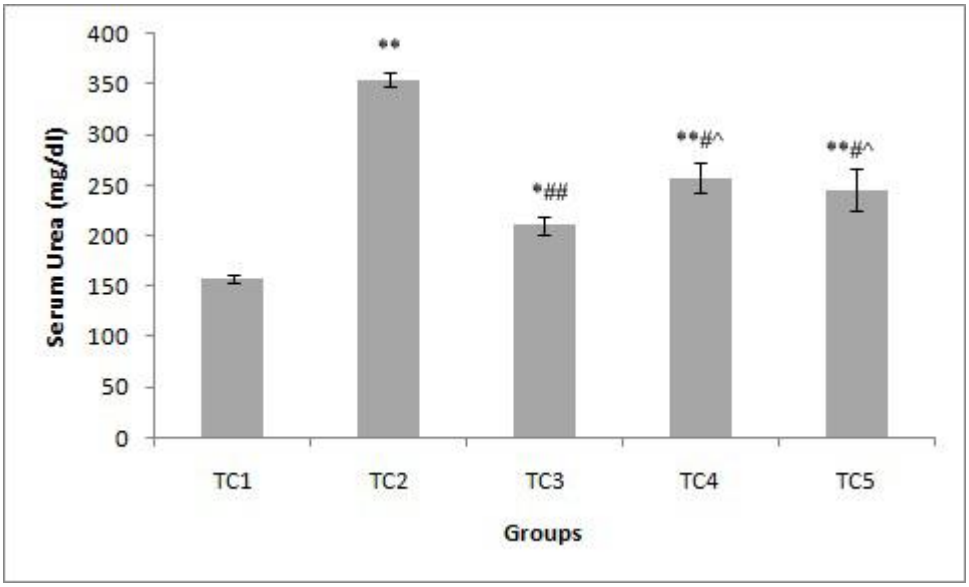


Figure 4.17: Content of Serum Urea after the treatment period of 28 days. $^{*}p<0.05$, $^{**}p<0.01$: Indicate significant change in comparison to control group TC1. $^{\#}p<0.05$, $^{\#\#}p<0.01$: Indicate significant change in comparison to hyperoxaluric group TC2. $^{\wedge}p<0.05$, $^{\wedge\wedge}p<0.01$: Indicate significant change in comparison to positive control group TC3.

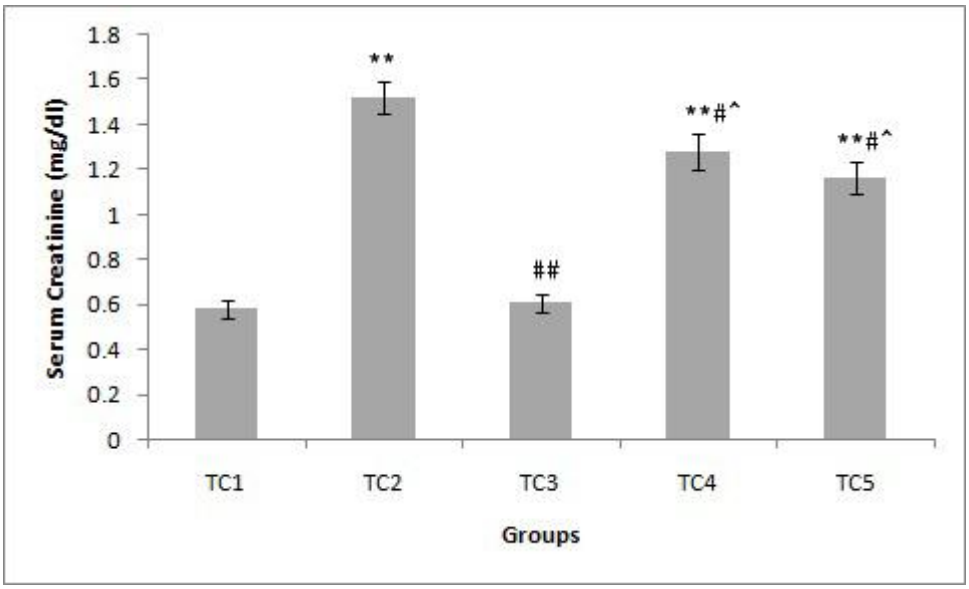


Figure 4.18: Content of Serum Creatinine after the treatment period of 28 days. $^{*}p<0.05$, $^{**}p<0.01$: Indicate significant change in comparison to control group TC1. $^{\#}p<0.05$, $^{\#\#}p<0.01$: Indicate significant change in comparison to hyperoxaluric group TC2. $^{\wedge}p<0.05$, $^{\wedge\wedge}p<0.01$: Indicate significant change in comparison to positive control group TC3.

4.3.2.4 Creatinine Clearance

Creatinine clearance was calculated using serum and urine creatinine content and as represented in Figure 4.19 for a treatment period of 28 days. Figure shows that the animals in the hyperoxaluric group TC2 showed a marked decrease in creatinine clearance (63%) as compared to control animals (Group TC1) significantly ($p<0.01$). Concurrent administration of cystone along with the exposure of EG + NH_4Cl restored the levels to normal with a decrease of 13% in comparison to group TC1. 50 mg/kg body wt dosage of aqueous of *Tribulus terrestris* was found to be less effective showing an increase of 60% w.r.t. TC1 group animals though, 100 mg/kg body wt plant dose which decreased the creatine clearance rate by 49% in comparison to control group TC1, proved to be effective in increasing the creatinine clearance rate in comparison to group TC2 animals.

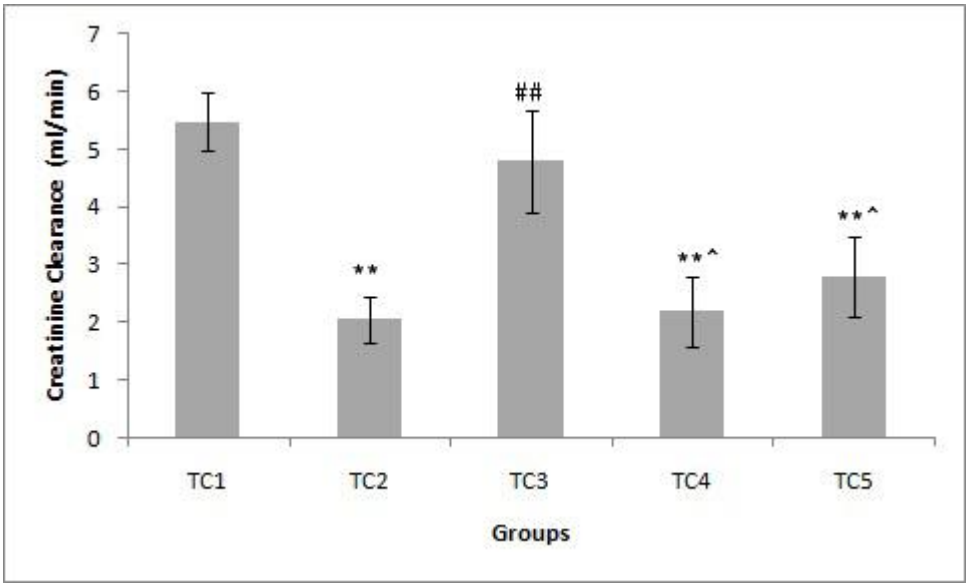


Figure 4.19: Effect on Creatinine Clearance after the treatment period of 28 days. $^{*}p<0.05$, $^{**}p<0.01$: Indicate significant change in comparison to control group TC1. $^{\#}p<0.05$, $^{\#\#}p<0.01$: Indicate significant change in comparison to hyperoxaluric group TC2. $^{\wedge}p<0.05$, $^{\wedge\wedge}p<0.01$: Indicate significant change in comparison to positive control group TC3.

4.3.2.5 Histopathological Analysis of renal tissue

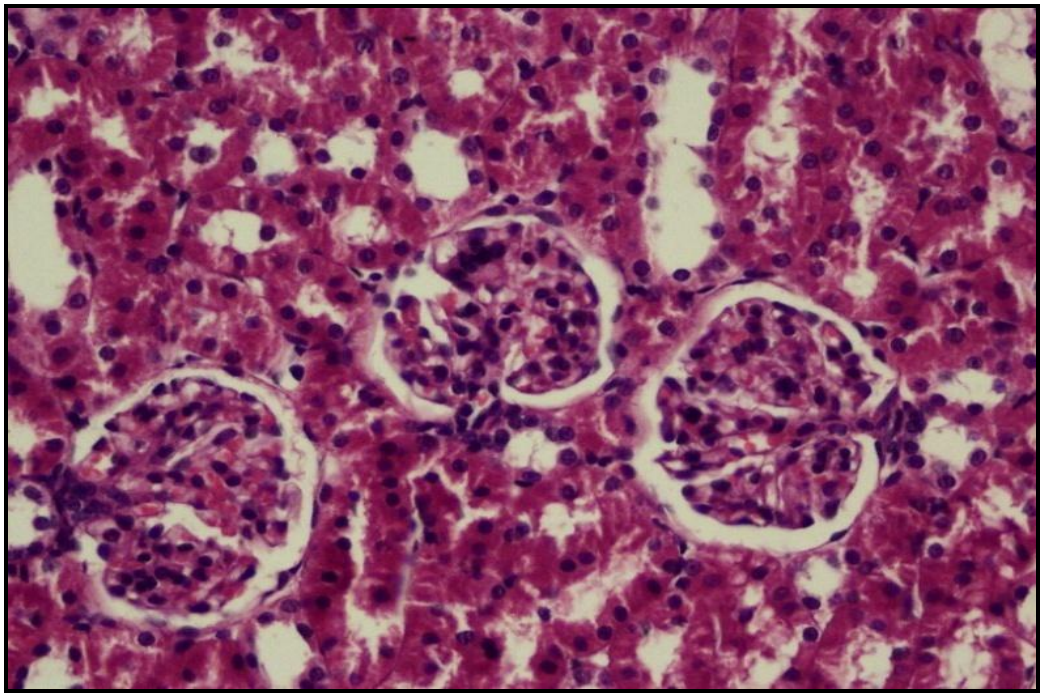
As shown in Figure 4.20, the histopathological observations of kidney showed normal structure and architectural intactness without any apparent damages in control (TC1). On histopathological examination, the ethylene glycol and ammonium chloride induced lithiatic group (TC2) showed the presence of polymorphic irregular CaOx crystals in lumina of tubules accompanied by edema and cast formation which causes dilation of proximal tubules along with interstitial infiltration. Severe atrophied glomeruli are observed on exposure to EG and NH_4Cl.

On administration of cystone, there was restoration to normal renal architecture with glomerulus regaining their shape, though infiltration was still observed. Treatment with 50 mg/kg body wt and 100 mg/kg body wt aqueous plant extract (group TC4 and TC5 respectively); curative effect was observed in a dose dependent manner. There was mild appearance of edema and dilation in tubules along with interstitial inflammation in group TC4 animals, though group TC5 animals observed intactness in glomerular structure. Tubular necrosis is also decreased in a dose dependent manner.

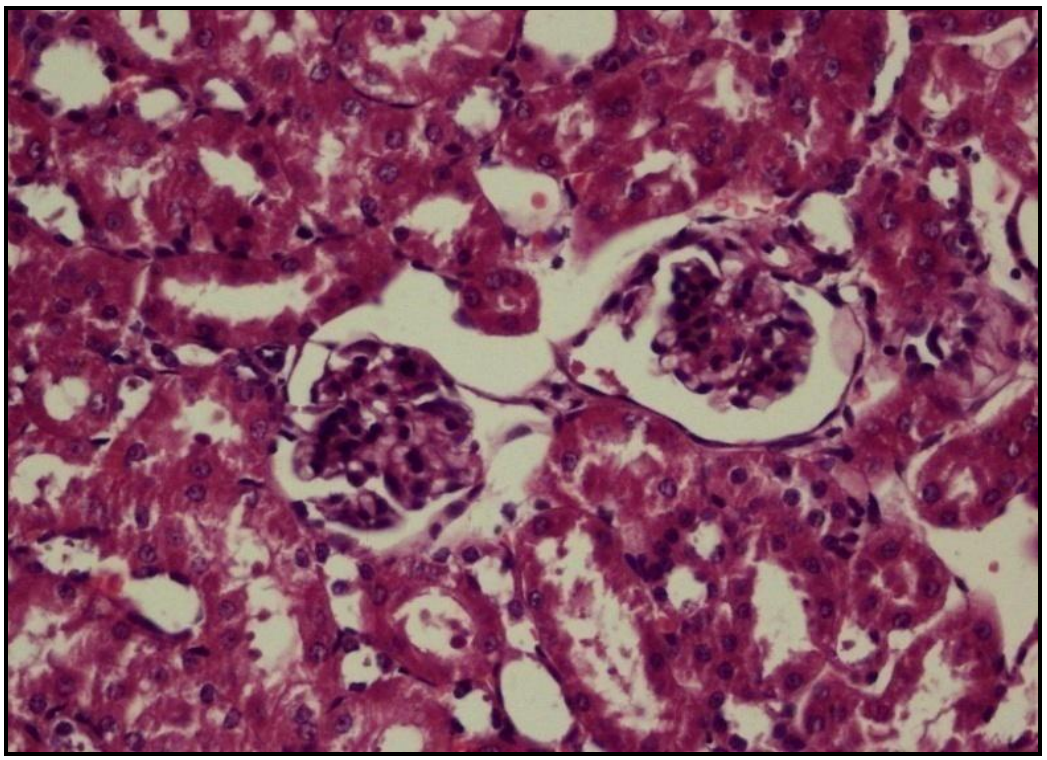
Table 4.2 illustrates the various histopathological features of the kidneys of rats of different treatment groups. Grades are based on observation of renal damage as visualized under light microscopic: "-" Not detected; "+" Mild; "++" Moderate; "+++" Severe.

Histopathological Feature	**Control (TC1)**	**Hyperoxaluric (TC2)**	**Cystone treated (TC3)**	**Dose 1 of *T. terrestris* (TC4)**	**Dose 2 of *T. terrestris* (TC5)**
Glomerular congestion	-	+++	+	++	+
Peritubular congestion	-	+++	+	+++	++
Epithelial desquamations	-	+++	+	+++	++
Vascular dilation	-	+++	-	++	+
Necrosis	-	++	+	+	+

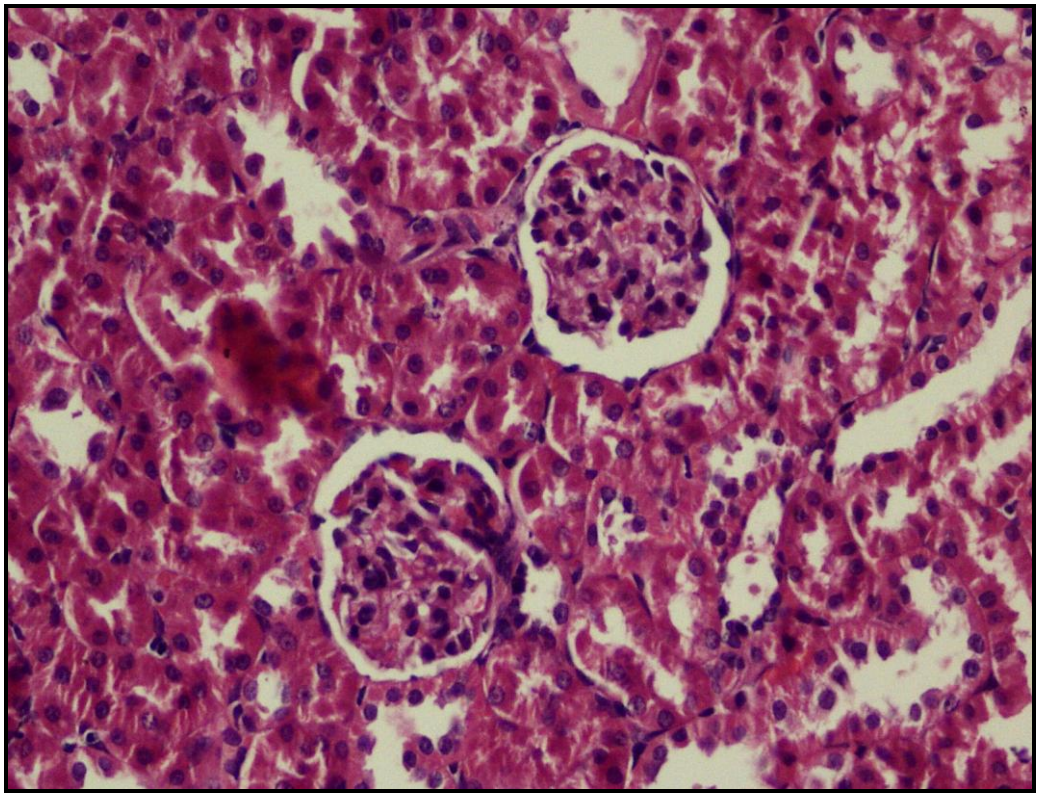
Table 4.2: Effect on renal histopathology in control and experimental animals after the treatment period of 28 days



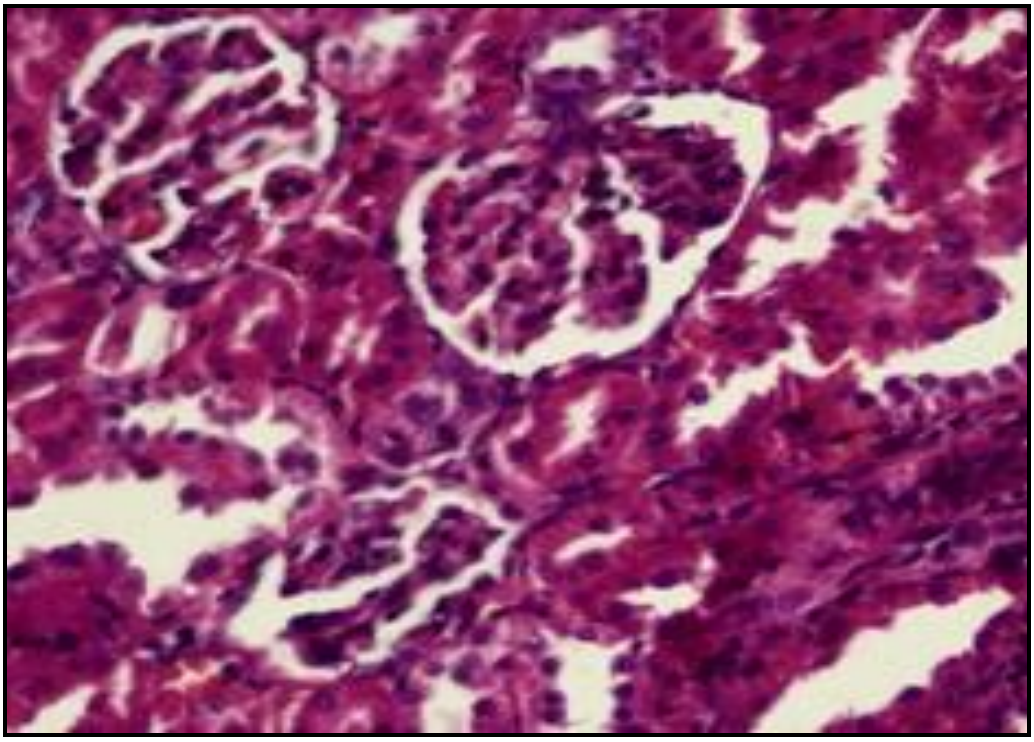
Group TC1



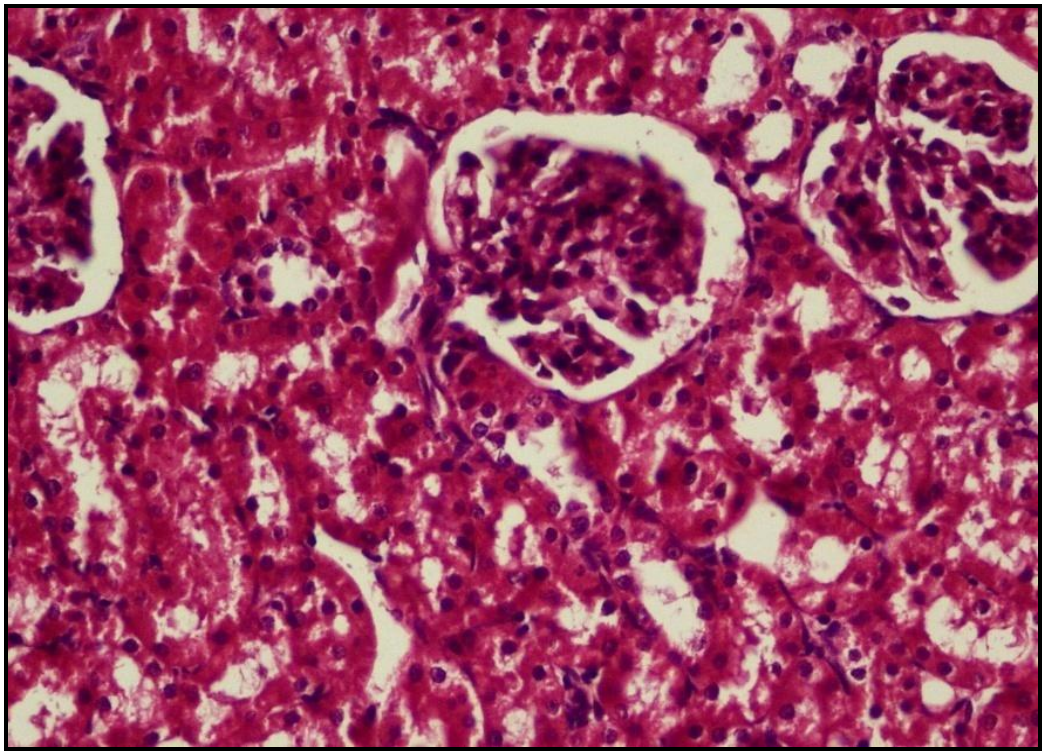
Group TC2



Group TC3



Group TC4



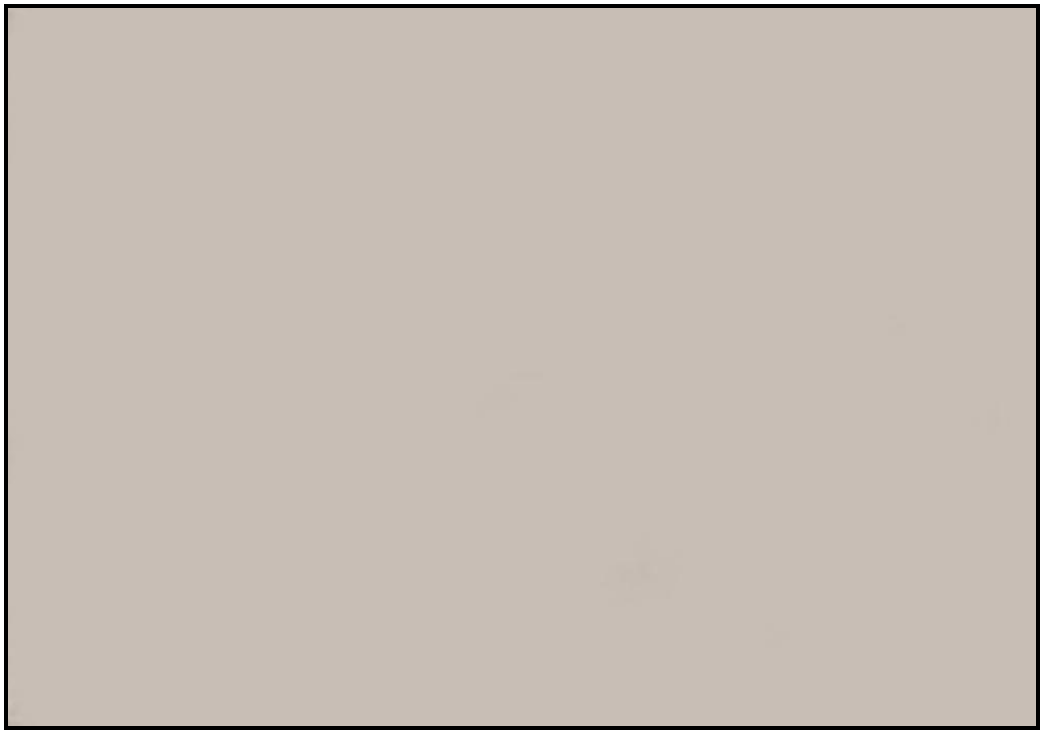
Group TC5

Figure 4.20: Renal histopathology of rats under light microscope given treatment for 28 days. Magnification: 400X. Group TC1: Untreated, Group TC2: Hyperoxaluric, Group TC3: Cystone treated, 750 mg/kg body wt, Group TC4: Dose 1 of *Tribulus terrestris*, 50 mg/kg body wt, Group TC5: Dose 2 of *Tribulus terrestris*, 100 mg/kg body wt

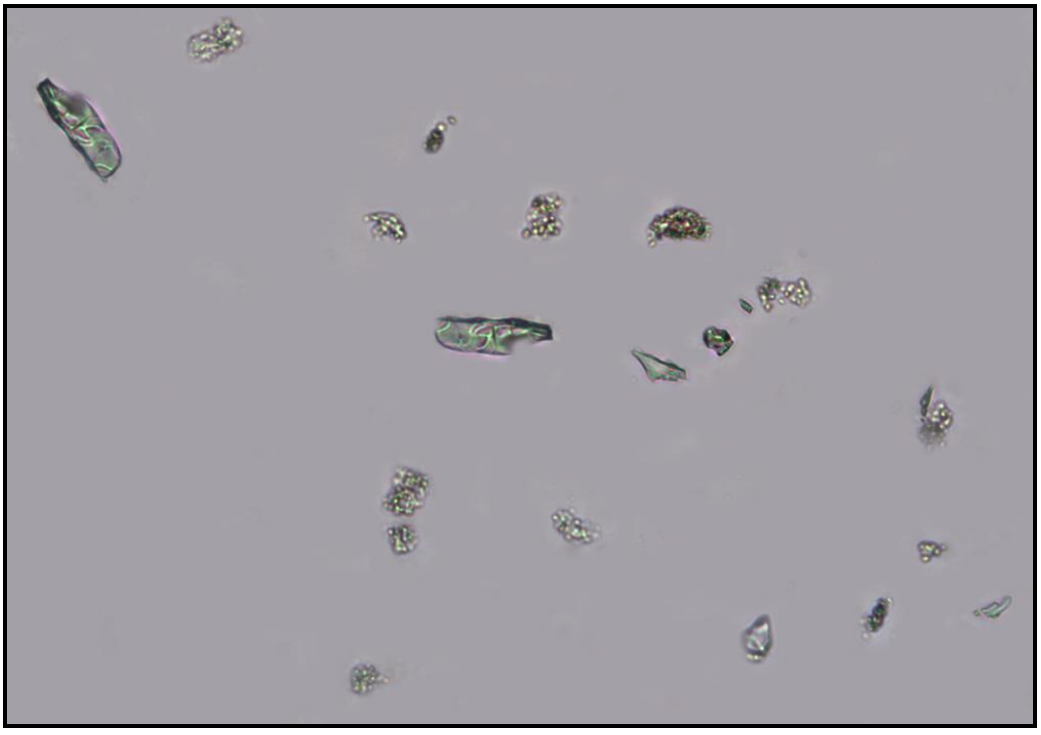
4.3.2.6. Polarization Microscopy of Urine

Polarization light microscopic observation revealed that urine of control group rats was more or less devoid of any crystal (TC1) whereas in urolithiatic group rats, the urine sample revealed presence of abundant crystals (TC2), which included CaOx monohydrate, CaOx dihydrate and triple phosphate as shown in Figure 4.21. Crystals are observed in the form of aggregates too.

In group TC3 animals, which were given a treatment of Cystone, 750 mg/kg body wt, very few crystals of CaOx were observed. Groups TC4 and TC5, administered with 50 mg/kg body wt and 100 mg/kg body wt of aqueous *Tribulus terrestris* respectively along with lithogenic treatment, and exhibited a significant decrease in the number of crystals in a dose dependent pattern.

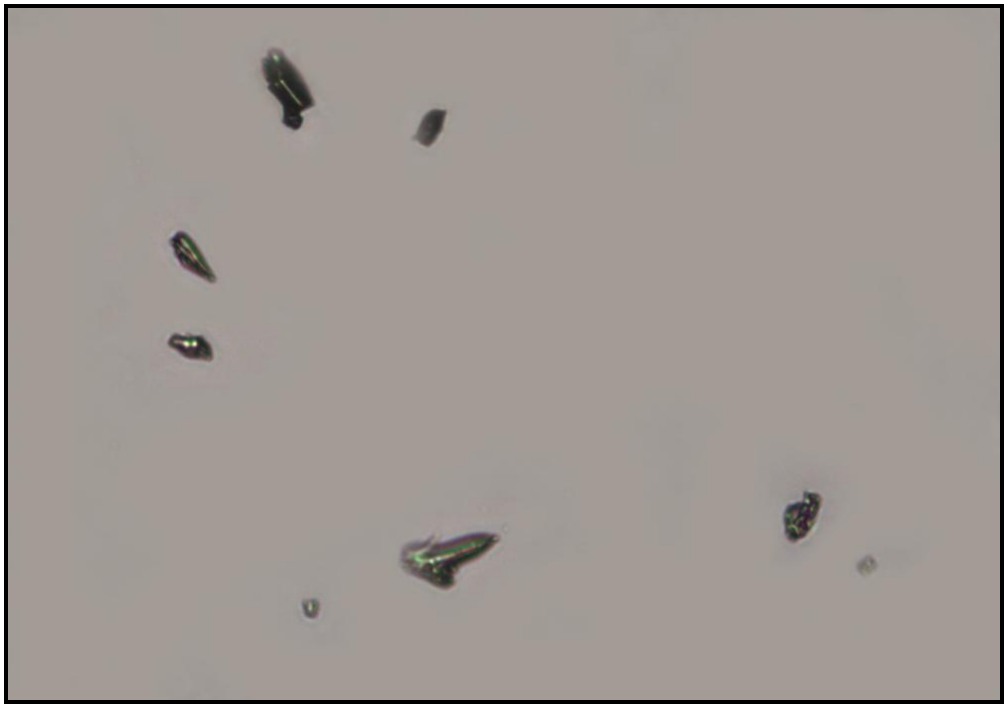


Group TC1

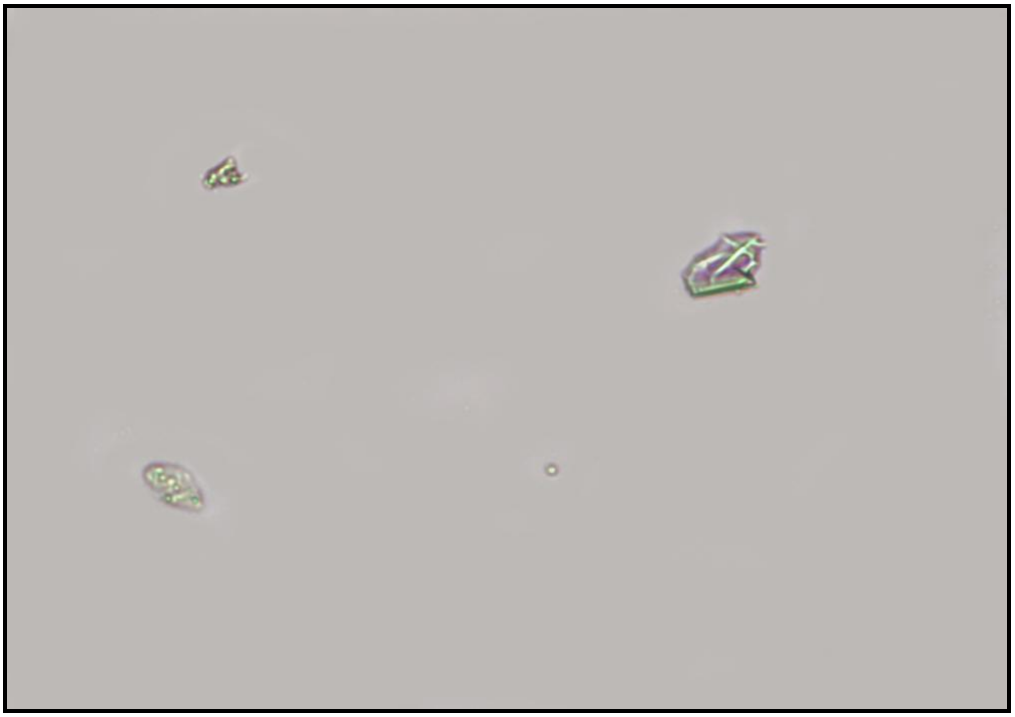


Group TC2

Group TC3



Group TC4



Group TC5

Figure 4.21: Polarization Micrographs of rat's urine given treatment for 28 days. Magnification: 100X. Group TP1: Untreated, Group TP2: Hyperoxaluric, Group TP3: Cystone treated, 750 mg/kg body wt, Group TP4: Dose 1 of *Tribulus terrestris*, 50 mg/kg body wt, Group TP5: Dose 2 of *Tribulus terrestris*, 100 mg/kg body wt

4.4 Purification of antilithiatic protein from *Tribulus terrestris*

The relationship of proteins to calculogenesis with regards to their effect as inhibitors or promoters during stone formation is proposed in literature. Abnormality in function and/or expression levels of proteins has been proposed to be associated with stone formation. While some progress has been made in the characterization of proposed inhibitors, their exact mechanism of action is still to be elucidated. Identification of additional stone-inhibitory proteins was hampered in the past by limitations in protein isolation methods, difficulties with protein handling, stability and lack of characterization instrumentation of proteins. The identification of additional stone inhibitors would increase the understanding of the pathogenesis and pathophysiology of nephrolithiasis.

Tribulus terrestris, commonly called gokhru, was found to possess biomolecules inhibiting CaOx crystallization *in vitro*, protection towards oxalate induced renal epithelium injury in NRK-52E and restored hyperoxaluric conditions to normalcy to a great extent *in vivo*. Proteins extracted in 50mM Tris-Cl buffer were found to possess anticalcifying potency. So, a conventional protein purification method including ammonium sulphate precipitation, ion exchange chromatography and molecular sieve chromatography was used to purify the proteins. The schematic representation used for the purification of the most potent antilithiatic protein is shown in Figure 3.3. The fractions obtained after each step of purification were assayed for their antilithiatic properties by employing *in vitro* calcium oxalate crystal growth assay. The protein content was estimated by Lowry's method and extent of purity of each active fraction was determined by SDS-PAGE analysis.

4.4.1 Tris-Cl Buffer extract

Proteins were extracted from the fruits of *Tribulus terrestris* with 50mM Tris-Cl buffer (pH 7.4), containing 0.25M NaCl, 1mM PMSF and 0.01% sodium azide for 24hrs with gentle stirring at 4^{o}C. Concentration of proteins quantified from Tris-Cl extract was 2.6mg/ml having an inhibitory potency of 37.56% with respect to control for CaOx crystal growth.

4.4.2 Ammonium sulphate precipitation

Tris-Cl crude protein extract was fractionated using ammonium sulphate precipitation. The pellets obtained were re-dissolved in the same buffer and dialyzed them through amicon tubes (cut off: 3kDa) for desalting. Table 4.3 illustrates the data obtained throughout the ammonium sulphate precipitation procedure with regards to protein concentration and CaOx inhibitory activity after dialysis. As shown in the table, the precipitates obtained between 40-60% ammonium sulphate contained maximum concentration of the protein with maximum inhibition of CaOx crystal growth, closely followed by the precipitates obtained between 60-80% ammonium sulphate in respect to protein concentration and inhibitory activity of CaOx growth. Almost all fractions obtained after ammonium sulphate precipitation showed some extent of inhibition towards CaOx *in vitro* assay system.

4.4.3 Anion exchange chromatography

The desalted 40-60% ammonium sulphate precipitation fraction was loaded on a Macro Prep® 25Q Strong anion exchanger (Bio-Rad Laboratories) column to separate the proteins present in the sample on the basis of their charge. Figure 4.22 shows the elution profile of anion exchange chromatography of 40-60% ammonium sulphate precipitation fraction. The blue line in the figure represents the absorbance (A.U.) of

proteins at 280nm present in the sample and the red line represents the conductivity (mS/cm). The fractions under the peak were pooled and the protein content was determined. Table 4.4 depicts the inhibitory potential of two most potent fractions thus obtained. The fraction, P3 which was obtained between conductivity 71.13 mS/cm and 85.99 mS/cm, had protein concentration of 100µg/ml and showed the maximum inhibitory activity (51.6%) towards CaOx growth.

Precipitation Range (%)	**Concentration of protein**	**%age inhibition of CaOx growth**
0-20	4.03 mg/ml	6.89
20-40	4.86 mg/ml	20.1
40-60	**7.9 mg/ml**	**38.61**
60-80	6.89 mg/ml	35.23
>80	5.74 mg/ml	10.67

%age inhibition of CaOx represents results as mean of three independent experiments

Table 4.3: Extent of inhibition of CaOx crystals growth assay after ammonium sulphate precipitation of *Tribulus terrestris* crude protein extract

Peaks after Anion Exchanger	**Pooled Fractions (minutes)**	**%age inhibition of CaOx growth**
P1	80-90	12.78
P3	**115-130**	**51.61**

%age inhibition of CaOx represents results as mean of three independent experiments

Table 4.4: Extent of CaOx inhibitory potential of fractions obtained after anion exchange chromatography

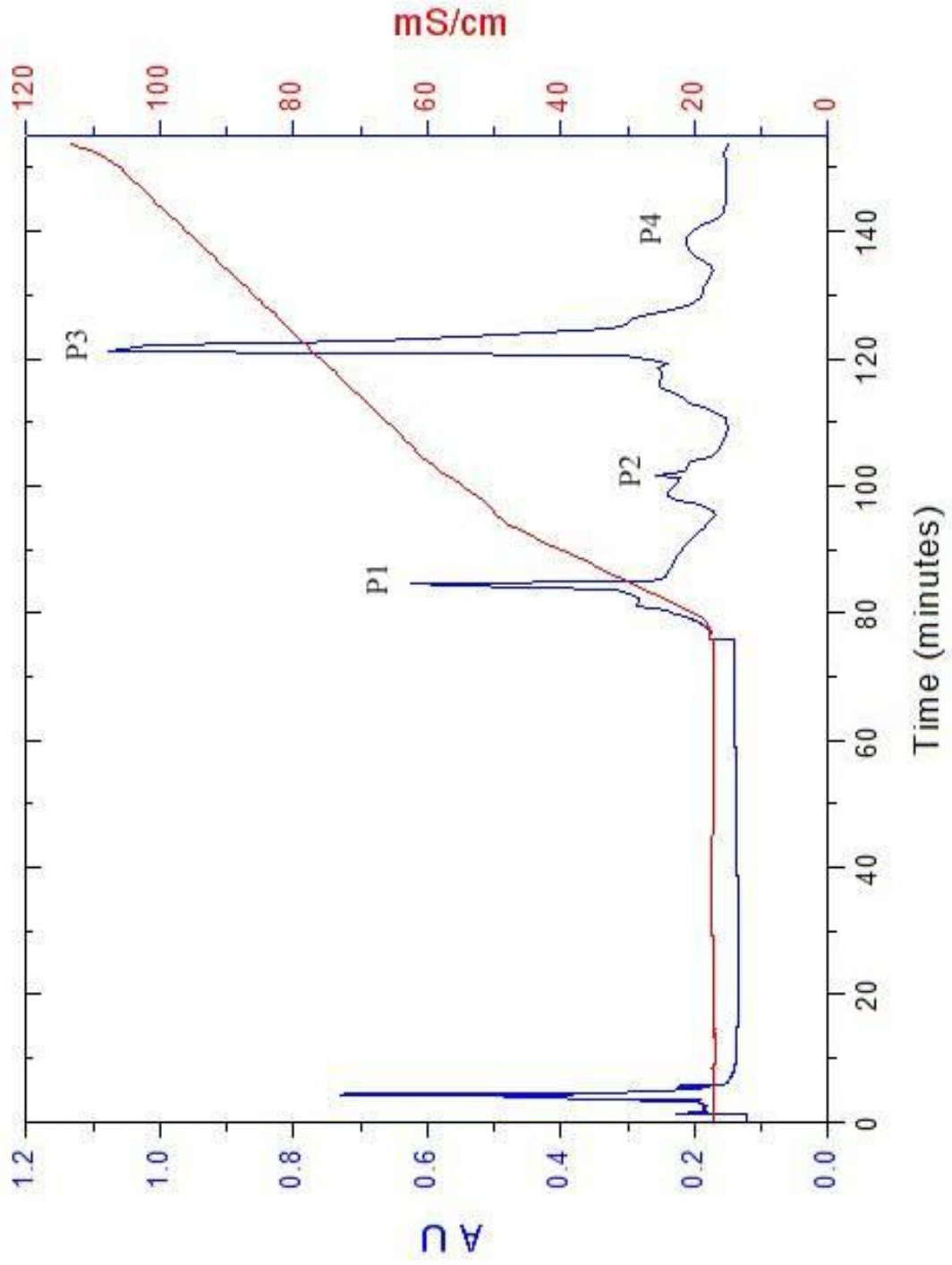


Figure 4.22: Elution profile of the protein sample loaded on anion exchange chromatography column after ammonium sulphate precipitation. The eluting peaks were detected at 280nm

4.4.4 SDS-PAGE of active peak

The most active peak i.e. peak P3 which was eluted between 115mins to 130mins with a conductivity range of 71.13 mS/cm and 85.99 mS/cm were analyzed by SDS PAGE. The gel picture is shown in Figure 4.23 which revealed two bands signifying it as a mixture of proteins. The figure is also showing the SDS-PAGE of the most active fraction from ammonium sulphate precipitation, 40-60% after dialysis.

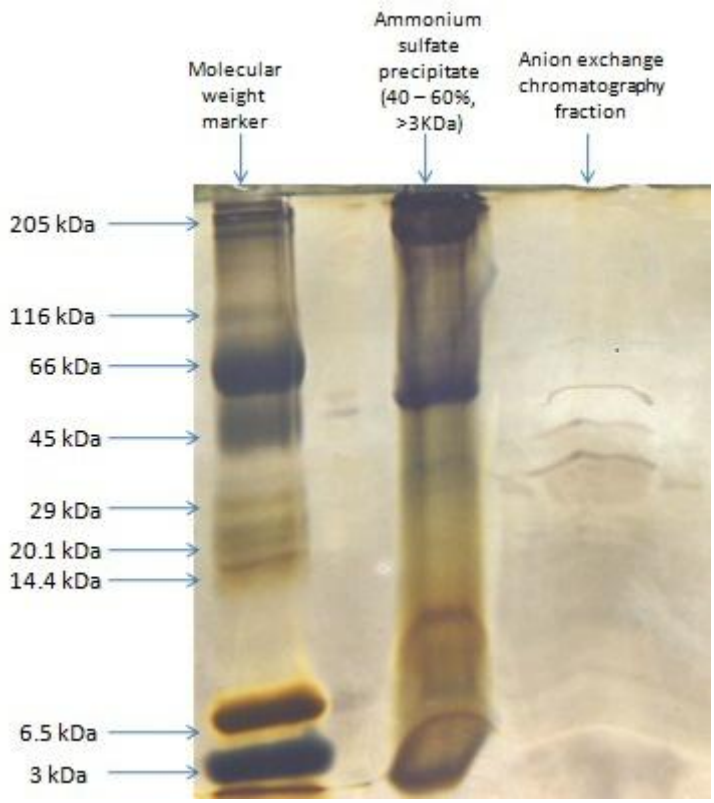


Figure 4.23: SDS-PAGE analysis of the peak P3 (115-130 min) after anion exchange chromatography

4.4.5 Molecular sieve chromatography

The pooled sample that contained the highest inhibitory activity after anion exchange chromatography, peak P3, was dialysed against 50mM Tris-Cl containing 50mM NaCl and loaded on a Bio gel® P-100 gel (Medium, 90-180µm) Molecular sieve

support (Bio-Rad Laboratories) column in order to separate the mixture of proteins based on their molecular size. Figure 4.24 shows the elution profile of the molecular sieve chromatography. The complete run of the sample was of 3000min with a flow rate of 0.1ml/min. The proteins were detected spectrophotometrically at 280nm. In this entire run, a single peak P1* was obtained. Fractions under that peak were pooled and quantification of proteins was done by Lowry's estimation. 82.53 µg/ml of the protein was obtained possessing an inhibitory potency of 78.26%.

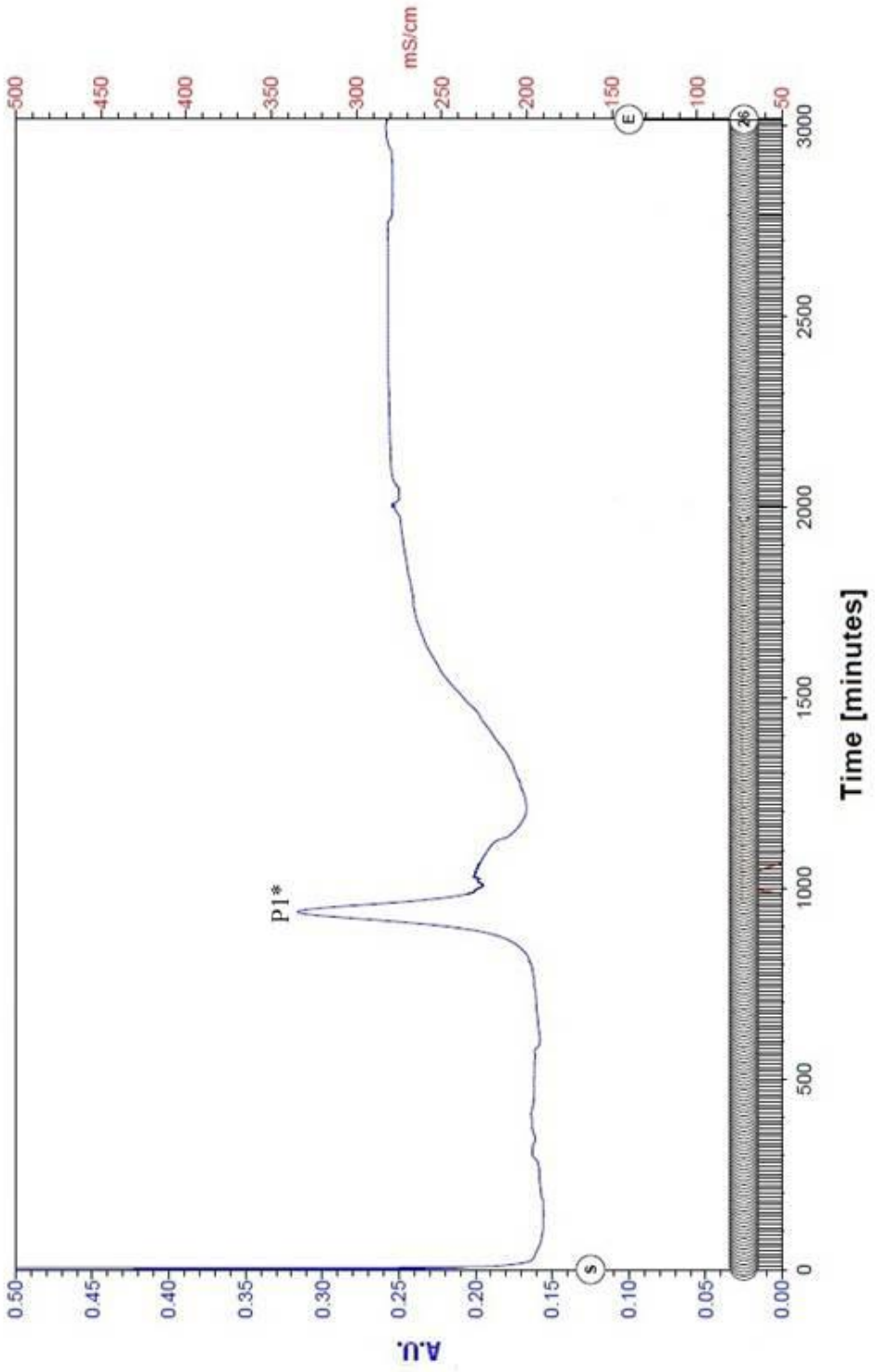


Figure 4.24: Elution Profile of protein sample P3 loaded on molecular sieve support after anion exchange chromatography. The eluting proteins were detected at 280nm

4.4.6 SDS-PAGE of active peak

As a single peak was obtained during molecular sieve chromatography, SDS-PAGE analysis of the protein sample was done to test the homogeneity of the sample. Figure 4.25 illustrates the SDS-PAGE analysis of the active fraction, P1*, which depicts a single band showing that protein is pure.

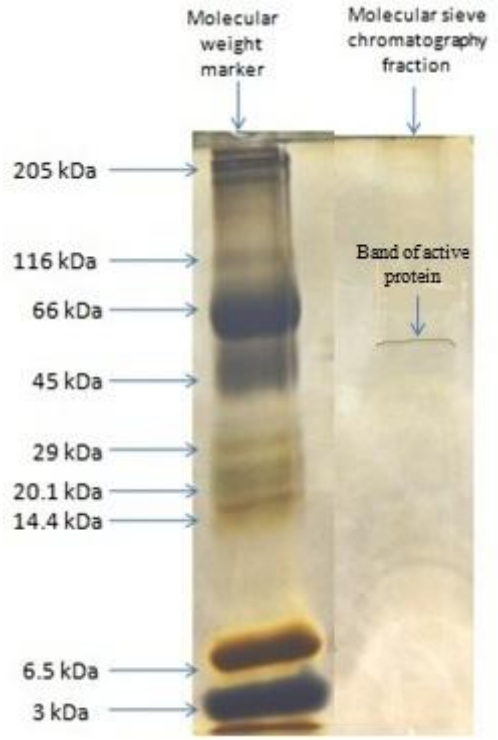


Figure 4.25: SDS-PAGE analysis of the peak P1* pooled after molecular sieve chromatography

4.4.7 Summary of purification of antilithiatic protein

The various steps used for purification of an antilithiatic protein from the fruits of *Tribulus terrestris* and the data obtained throughout the purification process are summarized in Table 4.5.

Purification Steps	Amount of protein	Yield (%)	%age Inhibition of CaOx growth
Tris-Cl Buffer Extract	286 mg	100	37.56
40-60% Ammonium sulphate precipitate (dialysed)	118.5 mg	41.4	38.61
Anion Exchange Chromatography	1.5 mg	0.5	51.61
Molecular Sieve Chromatography	0.74 mg	0.3	78.26

Table 4.5: Summary of the purification of inhibitory protein from the fruits of *Tribulus terrestris*.

Data refers to the protein obtained through different stages of purification from 100 g of powdered fruits of *Tribulus terrestris*. %age inhibition of CaOx represents results as mean of three independent experiments.

4.5 Characterization of the purified antilithiatic protein

4.5.1 Total amino acid composition of Tribulus terrestris antilithiatic protein (TTP)

Amino acid analysis of TTP was done by HPLC based method of Elkin and Wasynczuk. The acid hydrolysis of TTP resulted in the disintegration of the protein into free amino acids. These free amino acids on analysis by HPLC using a silica abased column, gave peaks corresponding to their elution time. These peaks were identified using elution time with standards of every amino acid and the area under the peak gave the corresponding concentration of that amino acid.

Figure 4.26 shows the elution profile of the acid hydrolyzate of TTP protein after HPLC. From the figure, it can be inferred that the protein is tyrosine rich as a result of which peaks of all other amino acids are not visualised.

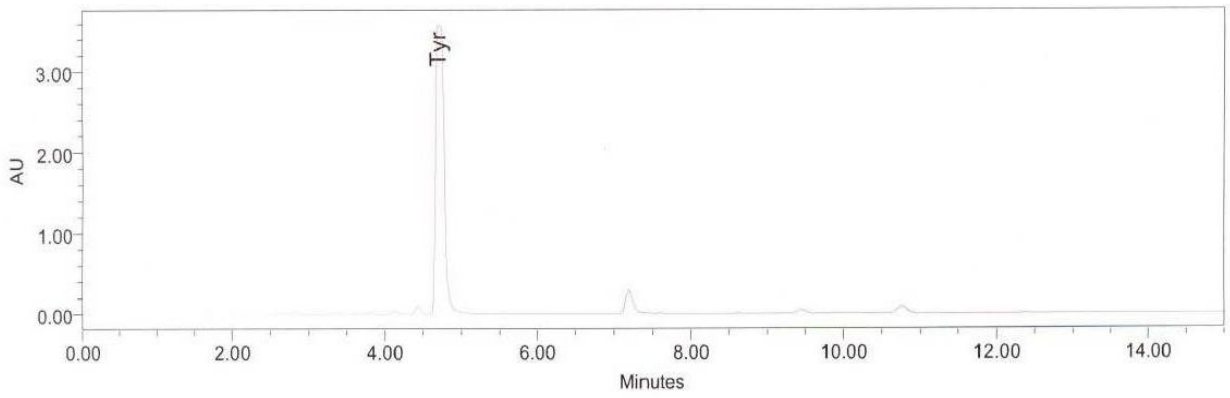


Figure 4.26: HPLC profile of Amino Acid analysis

4.5.2 Peptide mass fingerprinting

The trypsin digested of TTP was loaded over MALDI-TOF MS for peptide mass finger printing. MALDI-TOF analysis spectrum of *Tribulus terrestris* protein is shown in Figure 4.27. MALDI-TOF MS segregate the peptides on the basis of their mass to charge ratios. As a result each peptide moves with different speed in the

analyzer and depending on their time of flight, peptides reach at different times to the detector. Figure 4.27 shows various peaks with their corresponding m/z ratios.

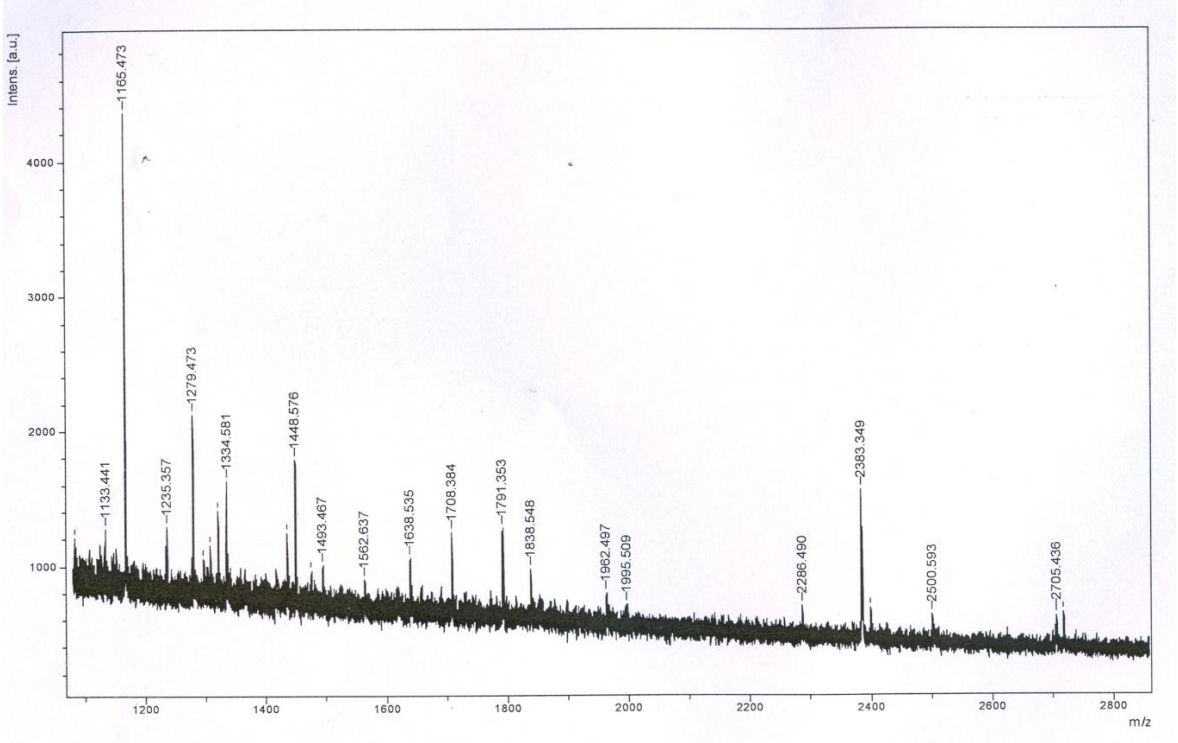


Figure 4.27: The peptide mass fingerprinting by MALDI-TOF MS obtained from trypsinised *Tribulus terrestris* protein

4.5.3. Peptide matching

The m/z ratios of all peptides as observed by MALDI-TOF MS were loaded in MASCOT search engine. MASCOT search engine compares m/z ratios obtained after MALDI-TOF MS with all proteins present in the database under the parameters stated in section 3.5.4. MASCOT search engine showed highest similarity (score: 41) of this protein with carotenoid cleavage dioxygenase 7 (CCD7) of *Arabidopsis thaliana* (CCD7_ARATH) having 17% sequence coverage. Proteins with probability based MOWSE scores exceeding their threshold ($p<0.05$) were considered to be positively identified. Figure 4.28 presents the results after MASCOT search.

Match to: **CCD7_ARATH** Score: **41** Expect: **2.3**
Carotenoid cleavage dioxygenase 7, chloroplastic OS=Arabidopsis thaliana GN=CCD7 PE=1 SV=1

Nominal mass (M_r): 69979; Calculated pI value: 6.01
NCBI BLAST search of CCD7_ARATH against nr

Taxonomy: Arabidopsis thaliana
Fixed modifications: Carbamidomethyl (C)
Variable modifications: Oxidation (M)
Cleavage by Trypsin: cuts C-term side of KR unless next residue is P
Number of mass values searched: **26**
Number of mass values matched: **10**
Sequence Coverage: **17%**
Matched peptides shown in **Bold Red**

```
  1 MSLPIPPKFL PPLKSPPIHH HQTPPPLAPP RAAISISIPD TGLGRTGTIL
 51 DESTSSAFRD YQSLFVSQRS ETIEPVVIKP IEGSIPVNFP SGTYYLAGPG
101 LFTDDHGSTV HPLDGHGYLR AFHIDGNKRK ATFTAKYVKT EAKKEEHDPV
151 TDTWRFTHRG PFSVLKGGKR FGNTKVMKNV ANTSVLKWAG RLLCLWEGGE
201 PYEIESGSLD TVGRFNVENN GCESCDDDDS SDRDLSGHDI WDTAADLLKP
251 ILQGVFKMPP KRFLSHYKVD GRRKRLLTVT CNAEDMLLPR SNFTFCEYDS
301 EFKLIQTKEF KIDDHMMIHD WAFTDTHYIL FANRVKLNPI GSIAAMCGMS
351 PMVSALSLNP SNESSPIYIL PRFSDKYSRG GRDWRVPVEV SSQLWLIHSG
401 NAYETREDNG DLKIQIQASA CSYRWFDFQK MFGYDWQSNK LDPSVMNLNR
451 GDDKLLPHLV KVSMTLDSTG NCNSCDVEPL NGWNKPSDFP VINSSWSGKK
501 NKYMYSAASS GTRSELPHFP FDMVVKFDLD SNLVRTWSTG ARRFVGEPMF
551 VPKNSVEEGE EEDDGYIVVV EYAVSVERCY LVILDAKKIG ESDAVVSRLE
601 VPRNLTFPMG FHGLWASD
```

Figure 4.28: Using MASCOT search engine (www.matrixscience.com), peptide masses from TTP showed 17% sequence coverage

4.5.4. Putative function and Domain Prediction

The BLASTp analysis of CCD7 of *Arabidopsis thaliana* showed its similarity with NP_182026.4 (9-cis-epoxycarotenoid dioxygenase [*Arabidopsis thaliana*]), ACY01408.1 (Carotenoid cleavage dioxygenase 7 [*Petunia* hybrid]), NP_001183928.1 (Carotenoid cleavage dioxygenase [*Zea mays*], ZP_01619401.1 (Retinal pigment epithelial membrane protein [*Lyngbya sp*]) showing that the purified protein belong to carotenoid cleavage dioxygenase family. The results reconfirm that CCD7 belongs to carotenoid cleavage dioxygenase family.

Figure 4.29 shows domains in the purified protein of *Tribulus terrestris* by ScanProsite. This protein has EF hand calcium binding domain from 228-240. The EF hand motif contains a helix-loop-helix topology, much like the spread thumb and forefinger of the human hand, in which the Ca^{2+} ions are coordinated by ligands within the loop as seen in Figure 4.30 (Lewit-Bentley and Rety, 2000). 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.

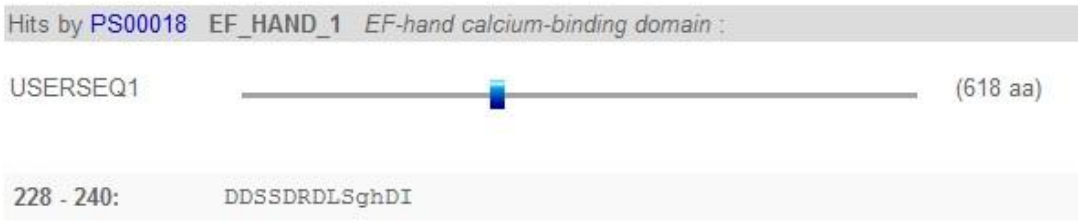


Figure 4.29: Domain identified in Carotenoid cleavage Dioxygenase 7 (CCD7) of *Arabidopsis thaliana* by ScanProsite. The sequence of the EF hand domain is represented.

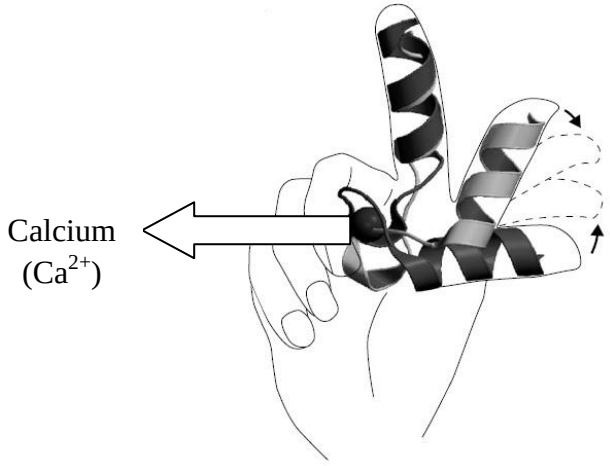


Figure 4.30: Schematic representation of EF Hand domain depicting change in conformation on binding of calcium

4.6 Protective effect of potent protein biomolecules of *Tribulus terrestris* on NRK-52E

4.6.1 Cell Viability

Figure 4.31 depicts the protective effect of the purified protein towards the renal tubular epithelial cells, NRK-52E. The oxalate induced a significant injury to the cells as it reduced the viability from 100% in the untreated cells (control) to 75.1%. The purified protein proved to be protective towards oxalate induced injury as it increased the viability significantly in a concentration dependent manner from 1µg/ml to 4µg/ml. The percentage viability obtained with the purified protein was comparable to the cystone (positive control). The percentage viability with 1µg/ml, 2µg/ml and 4µg/ml of the protein was 77.1, 80.9 and 85.9 respectively. The percentage viability obtained with 4µg/ml was quite comparable to that obtained from 50µg/ml crude aqueous extract of *Tribulus terrestris* as seen in Table 4.6.

4.6.2 Cell Injury

The release of lactate dehydrogenase (LDH) was estimated to check the cell injury (Figure 4.32). In the cells injured with the exposure of oxalate, LDH release was significantly high (217.9%). The LDH release was significantly reduced in the cells which had the exposure of different concentrations of the purified protein with oxalate in a concentration dependent manner to 115.9% with 4µg/ml protein sample. The LDH release in the presence of test protein was quite comparable with that of the positive control, cystone. LDH release with 50µg/ml of aqueous extract was found to be 110%, which is quite comparable to the percentage LDH release found on treating cells with just 4µg/ml purified protein (TTP) (Table 4.6).

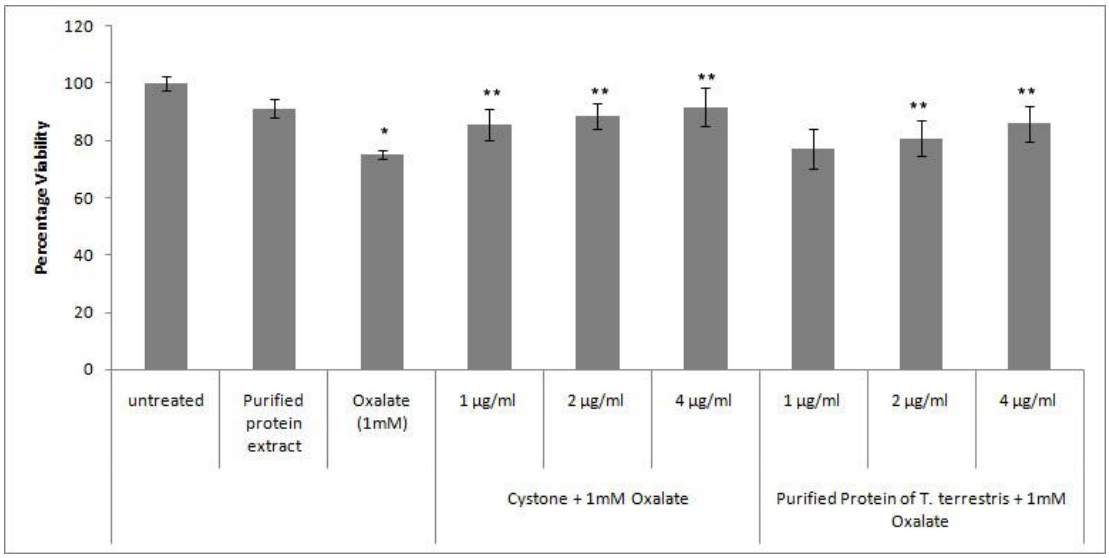


Figure 4.31: Effect of Cystone and the purified protein of *Tribulus terrestris* on the percentage viability of NRK-52E. Data are mean ± SEM of three independent observations. *$p<0.05$ *versus* untreated control, **$p<0.05$ *versus* oxalate control

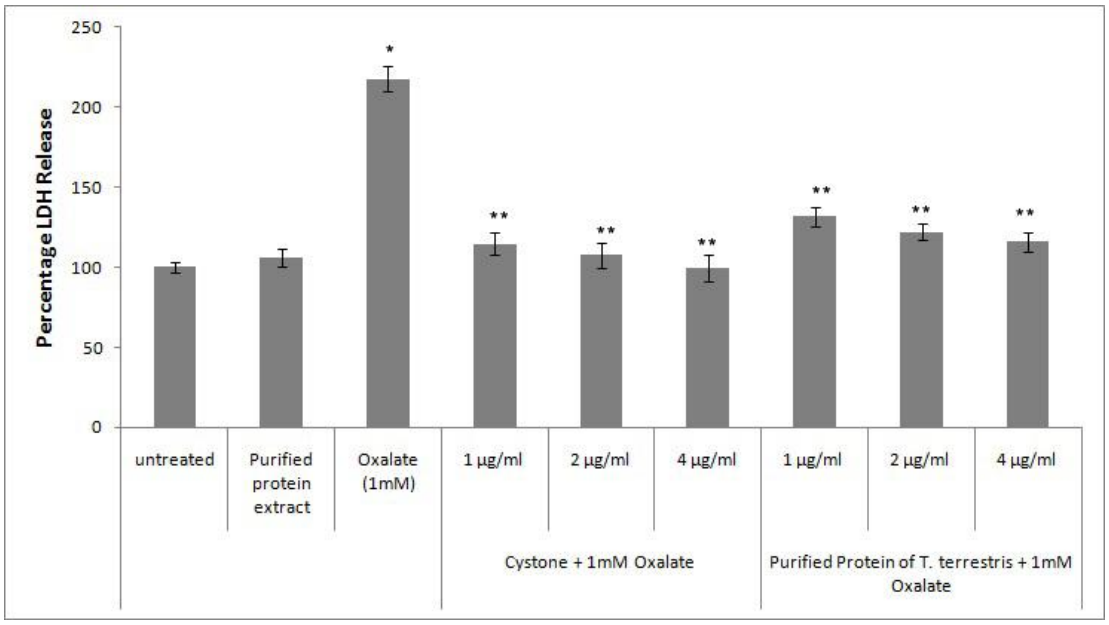


Figure 4.32: Effect of Cystone and the purified protein of *Tribulus terrestris* on the percentage LDH release of NRK-52E. Data are mean ± SEM of three independent observations. *$p<0.05$ *versus* untreated control, **$p<0.05$ *versus* oxalate control

	Percentage cell viability	Percentage LDH release
Untreated cells	100	100
Crude aqueous *T. terrestris* (AqE)	93.14	106.07
Purified Protein extract	91.2	106.2
1mM oxalate	75.1	217.9
1µg/ml cystone + 1mM oxalate	85.8	114.7
2µg/ml cystone + 1mM oxalate	88.5	107.4
4µg/ml cystone + 1mM oxalate	91.7	99.7
10µg/ml AqE + 1mM oxalate	81.62	126.54
25µg/ml AqE + 1mM oxalate	84.87	112.55
50µg/ml AqE + 1mM oxalate	89.14	109.76
1µg/ml purified protein + 1mM oxalate	77.1	131.7
2µg/ml purified protein + 1mM oxalate	80.9	122.2
4µg/ml purified protein + 1mM oxalate	85.9	115.9

Table 4.6: Comparison of the effect of Cystone (Positive control), AqE (Aqueous extract) and Purified protein of *Tribulus terrestris* on Percentage cell viability and Percentage LDH release of NRK-52E

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).

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), Uritone (Aimil Pharmaceuticals, India), Culdisol (Ganga Pharmaceuticals, India), Calcury (Charak Pharmaceuticals, India), Chandraprabhabati (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 (Beghalia *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 *Crataeva nurvala, 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 antiurolithiatic potency against CaP and CaOx *in vitro* crystallization as *Dolichos biflorus, Trachyspermum ammi* (Bijarnia *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 (Verkoelen *et al.*, 1997). 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 NH_4Cl (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% NH_4Cl 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 urea 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, γ-glutamyl transpeptidase, N-acetyl-β-D-glucosaminidase, cathepsin B, lysozyme, and lactate dehydrogenase; *2*) low-molecular-weight proteins: β_2-microglobulin, α_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 NH_4Cl 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 urea levels in hyperoxaluric rats (Bijarnia *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 (Kaissling *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 NH_4Cl 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 + NH_4Cl) 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 dioxygenases 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-cryptoxanthin) (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).

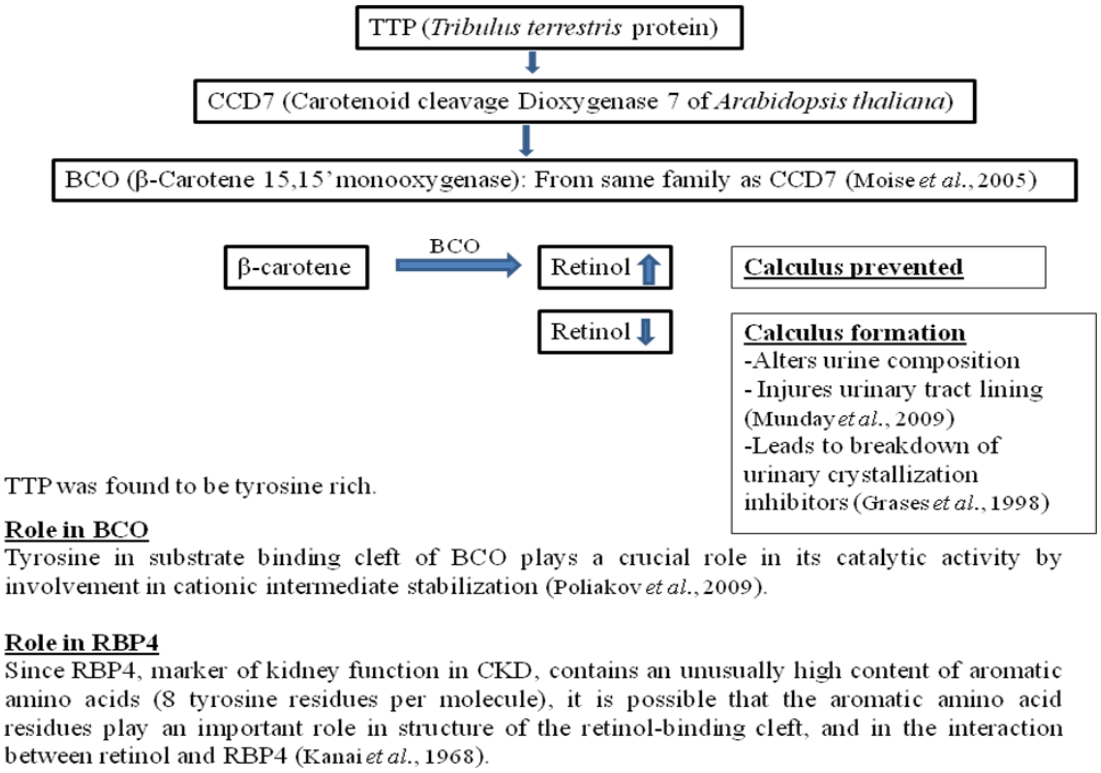


Figure 5.1: Hypothesis about the role of CCD7 in urolithiasis

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 (Lewit-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 A_2 ($cPLA_2$) is released upon the activation of MAP kinases and translocated to the cell membrane. $cPLA_2$ preferentially hydrolyses arachidonoyl phospholipids generating a number of byproducts including arachidonic acid and lysophospolipids. Exposure of MDCK cells to oxalate produces a time and concentration dependent increase in $cPLA_2$ activity (Jonassen *et al.*, 2003). Inhibition of $cPLA_2$ 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 (Lieske *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.

SUMMARY

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CONCLUSIONS

Urolithiasis, classified as one of the most painful disorders afflicting mankind since ancient times, affects 2 - 20% population worldwide and is the third most common affliction of the urinary tract (Johri *et al.*, 2010; Atmani, 2003). "Stone belt regions" of the world encompass countries of Middle East, North Africa, Mediterranean Regions, North Western states of India and Southern states of USA (Lopez and Hoppe, 2010). Prevalence of urinary stones differs in different parts of the world depending on various dietary and non-dietary factors with a prevalence rate of 15% in India (Rizvi *et al.*, 2002). One of the important phenomena that characterize urolithiasis is its high recurrence which is ~50% (Knoll, 2007) along with the increasing prevalence rate (Agarwal *et al.*, 2011).

The exact etiologic cascade of events which leads to urolithiasis is unknown. Hypotheses ranging from oxalate-induced renal injury to insufficient urinary inhibitors of calculogenesis, to nidus formation with epitaxy, have been proposed (Evan *et al.*, 2005). According to clinical and epidemiological studies, calcium oxalate (CaOx) followed by calcium phosphate (CaP) are the most frequently encountered crystalline components found in analyzed stones. Most stones do not contain one single crystal phase but rather a mixture of several different crystal phases (Prasad *et al.*, 2007). The concentration of ions (calcium, oxalate, urate, cystine, phosphate) in the tubular fluid, the level and activity of crystallization modulators (GAGs, pyrophosphate, citrate, nephrocalcin, uropontin, THP, urinary prothrombin 1) and the retention of crystals in the kidney are considered major determinants in the development of a stone (Verkoelen *et al.*, 1997; Khan and Kok, 2004).

Medical therapy is directed to relieve agonizing pain, dissolution and expulsion of stone and prevention of recurrence. Among the treatments used are extracorporeal

shock wave lithotripsy (ESWL), ureterorenoscopy (URS), percutaneous nephrolithotomy (PCNL) in conjugation with various drugs like NSAIDs, potassium citrate, allopurinol and dietary measures (Singh *et al.*, 2011, Sayer *et al.*, 2010). Exposure to ESWL may cause traumatizing effects of shock waves, persistent residual stone fragments and a possibility of infection leading to a decrease in renal function thereby posing serious problems to be taken into consideration (Atmani, 2003). Treatment and prevention of kidney stones has been considerably revolutionised during the last two decades by combination of dietary procedures, surgical treatments and medicaments but side effects and recurrence remain as problems to overcome. Thus, an adjunct to these conventional methods as phytotherapy is highly recommended.

The use of herbal medicine is becoming popular due to toxicity and side effects of allopathic medicines. Plant medicines are in great demand both in the developed as well as developing countries for the primary health care because of their wide biological and medicinal activities, higher safety margin and lesser costs. Many remedies have been employed during the ages to treat urinary stones. In the traditional system of medicine, most of the remedies were taken from plants and they proved to be useful though the rationale behind their use is not well established through systematic pharmacological and clinical studies except for some composite herbal drugs (Jethi *et al.*, 1983). The marketed composite herbal formulations, Cystone (Himalaya Drug Company, India), Neeri (Aimil Pharmaceuticals, India), Uriflow (Bioneutrix Labs), Uritone (Aimil Pharmaceuticals, India), Culdisol (Ganga Pharmaceuticals, India), Calcury (Charak Pharmaceuticals, India), Chandraprabhabati

(Baidyanath, India) and Culin Forte (Alopa Herbal) have been used worldwide to dissolve urinary calculi in kidney and urinary bladder.

Fruits of *Tribulus terrestris* (Zygophyllaceae) locally named as "gokhru" in India are commonly used by folklore to treat urolithiasis. It is an active component of various herbal drug formulations like Cystone, Neeri and Uriflow. Its antilithiatic potency is not well explored yet. It is proposed to be diuretic and also suppressed the isoenzyme pattern of renal LDH, another promoter of hyperoxaluria (Sangeeta *et al.*, 1994). 10% aqueous methanol extract of *T. terrestris* most effectively prevented encrustation of implanted glass beads in the bladder of rats (Anand *et al.*, 1994), though no compound from *T. terrestris* is yet purified and reported to possess antiurolithiatic potency. Recently, the antilithiatic potential of aqueous extract of *Tribulus terrestris* was tested against calcium oxalate monohydrate crystals *in vitro* and was found to be efficient using a double diffusion gel growth technique (Joshi *et al.*, 2005b). Thus, the present study was aimed at investigating the inhibitory potency of aqueous extract of *Tribulus terrestris* on calcium oxalate crystallization *in vitro*, validating the efficacy of the extract *in vivo* along with the isolation and characterization of new biologically active compound(s) from *Tribulus terrestris*. The whole work is summarized below:

1. *Tribulus terrestris* extract exhibited a concentration dependent inhibition of nucleation and the growth of CaOx crystals. *Tribulus terrestris* was found to release calcium and phosphate ions during demineralization of the preformed mineral phase effectively with a slight inhibition of calcium ions during initial mineral phase formation.
2. Reduction of oxalate induced injury by aqueous extracts of *Tribulus terrestris* was evaluated on NRK-52E w.r.t. LDH release and cell viability wherein the

extract of the plant prevented the injury induced by oxalate in a dose-dependent manner. The antioxidant potential of *T. terrestris* may contribute to the decrease in the free radicals released due to injury caused by oxalate thus suggesting the protective potential of *T. terrestris*. 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. Antilithiatic activity of aqueous extracts of *Tribulus terrestris* was also validated in rat urolithiatic model to evaluate its efficacy *in vivo*. The rats induced with ethylene glycol and ammonium chloride showed a marked elevation of renal injury marker enzymes viz. alkaline phosphatase (ALP) and lactate dehydrogenase (LDH). The enhanced urinary excretion of these renal injury marker enzymes in urolithiatic rats suggests damage to the brush border membrane of the renal tubules which appears to be associated with the retention and deposition of crystals in the kidney. ALP and LDH excretion was normalized following the administration of aqueous extract of *Tribulus terrestris* in urolithiatic rats in a concentration dependent manner. This shows that aqueous extract of *Tribulus terrestris* reduced renal injury which further provides sites for CaOx deposition in both prophylactic and curative regimen. During renal dysfunction, there was an accumulation of nitrogenous waste compounds viz. urea and creatinine. Clinically, creatinine clearance is a useful measure for determining renal functioning. Administration of *Tribulus terrestris* extract along with lithogenic treatment decreased both serum urea and creatinine in comparison to hyperoxaluric groups. Histopathology of

kidney tissues and polarization of urine samples further validate the results obtained from biochemical assays.

4. Purification of potential protein inhibitor of CaOx crystal growth from *Tribulus terrestris* was carried out by conventional bioactivity-guided protein purification steps *viz.* ammonium sulphate-precipitation, anion-exchange chromatography and molecular-sieve chromatography along with validation by SDS-PAGE at each step. Proteins present in *Tribulus terrestris* were found to have an ability to inhibit CaOx crystal growth and it was observed that this inhibitory ability increased with successive protein purification steps. A single band protein having MW ~ 60 kDa was purified employing the above mentioned steps.
5. The protein band was excised and subjected to in-gel tryptic digestion for MALDI-TOF analysis to obtain a peptide mass fingerprint (PMF). MASCOT analysis of the protein purified from *Tribulus terrestris* (TTP) revealed its homology with carotenoid cleavage dioxygenase 7 (CCD7) of *Arabidopsis thaliana*. CCD7, which belongs to the family of dioxygenases and cleaves β-carotene (provitamin A) to produce 10'-apo-β-carotenal. Another significant member of this family is β carotene 15, 15' monooxygenase (BCO) based on biochemical and amino acid sequence data. BCO catalyzes the first step in the synthesis of retinol from dietary carotenoids. The mechanism by which retinol (vitamin A) deficiency causes calculus formation is still unclear though there are various hypotheses like deficiency of retinol is involved in altering urine composition, injuring urinary tract lining and causing deficiency of various urinary crystallization inhibitors like pyrophosphate and phytate. 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 was impaired on site-directed mutagenesis of these residues. BCO leads to an increase in the amount of retinol by breakdown of β-carotene substantiating the role of tyrosine in its catalytic action. Since, CCD7 belongs 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, hence, 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. 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. It is common calcium binding helix-loop-helix motif. Most of the kidney stone inhibitory proteins like nephrocalcin, osteonectin and calgranulin have EF hand domain.

6. Purified protein from *Tribulus terrestris* also exhibited cytoprotective effect on NRK-52E w.r.t. cell viability and cell injury which was much more in comparison to the cytoprotective potency by aqueous extract. Polyanionic compounds coat the crystalline surface thus inhibiting the binding of the crystals to the cells. The purified protein from *Tribulus terrestris* (TTP) was anionic thus explaining the protective potency by increasing the cell viability and decreasing the LDH release in its presence.

The aqueous extract of *Tribulus terrestris* has been shown to possess an ability to inhibit CaOx crystallization *in vitro*. Besides this, extract also showed cytoprotective properties towards the NRK-52E cells by lowering down the LDH leakage and increasing the cell viability. The extract has a potent prophylactic and curative effect as it is effective in reducing the renal tissue injury, decreasing the crystal size thus facilitating easy expulsion of crystals and restoring normal kidney architecture. A plant protein, anionic in nature, from the fruits of *Tribulus terrestris* was shown to attain the ability of inhibiting CaOx crystallization *in vitro*. This protein had similarity with CCD7 of *Arabidopsis thaliana* which belongs to the family of proteins having significant role of tyrosine in its catalytic action known to form retinol that plays a significant role in preventing calculi formation. The presence of EF hand domain in this protein further signifies its calcium binding properties which is a feature of most of the kidney stone inhibitors. 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.

PATENT &

PUBLICATIONS

PATENT FILED

- Filed an Indian Patent entitled "A novel anticalcifying protein from *Tribulus terrestris*", Chanderdeep Tandon, Anshu Aggarwal, (Application no. 2248/DEL/2011)

INTERNATIONAL PUBLICATIONS

- Aggarwal A, Tandon S, Singla SK, Tandon C. Diminution of oxalate induced renal tubular epithelial cell injury and inhibition of calcium oxalate crystallization *in vitro* by aqueous extract of *Tribulus terrestris. International Brazilian Journal of Urology* 2010; 36: 480-489 [Impact factor: 1.4][Indexed in PubMed 'MEDLINE']
- Aggarwal A, Singla SK, Priyadarshini, Tandon C. *In vitro* studies on anticalcifying potency of *Tribulus terrestris*. *International Journal of Urology* 2010; 17: A211-A212. [Impact factor: 1.460] [Indexed in PubMed 'MEDLINE', SCOPUS]
- Aggarwal A, Singla SK, Tandon C. Urolithiasis: Phytotherapy as an adjunct therapy. *Alternate Medicine Review*. [Impact factor: 3.57] [Indexed in PubMed 'MEDLINE'] [Accepted]

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- Aggarwal A, Singla SK, Priyadarshini, Tandon C. *In vitro* studies on anticalcifying potency of *Tribulus terrestris*. *10th Asian Congress of Urology of the Urological Association of Asia,* Organized by Taiwan Urological Association, August 27 – 31, 2010, Taipei, Taiwan.

- Prophylactic effect of *Tribulus terrestris* fruits on experimentally induced urolithiasis in rats, Aggarwal A, Singla SK, Singh SK, Tandon C. *European Urology Supplements*. [Impact Factor: 2.139] [Indexed in PubMed 'MEDLINE', SCOPUS] [Accepted]
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