Studies on Antilithiatic properties of *Tamarindus indica* and *Terminalia arjuna*

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Abstract

Urinary stones are one of the oldest and the most common afflictions in humans. This disease has tormented humans since the earliest records of civilization. As many as 10.0% of men and 3.0% of women have a stone during their adult lives. Calcium containing stones are the most common comprising about 75% of all urinary calculi, which may be in the form of pure calcium oxalate (50%) or calcium phosphate (5%) and a mixture of both (45%). Although, there are many medical treatments available, yet not much progress has been made in curing the disease. Techniques like extracorporeal shock wave lithotripsy (ESWL), percutaneous nephrolithotomy (PCNL) and open surgery have many side effects like bleeding, pain related to passage of stone fragments, failure to fragment the stone etc., thereby requiring a possible need for additional or alternative interventions.

An alternative to avoid such complications is the use of phytotherapy. A number of plants have been mentioned in Indian Ayurvedic system which play a vital role in the treatment of kidney stones. In the present study, the inhibitory potency of *Tamarindus indica* fruit and *Terminalia arjuna* bark has been investigated on various stages of calcium phosphate mineralization and on nucleation and growth of calcium oxalate monohydrate both *in vitro* and *in vivo*.

Efficacy of *Tamarindus indica* was first checked *in vitro* by using aqueous

extract against calcium phosphate mineralization and calcium oxalate growth. Aqueous extract of *Tamarindus indica* not only proved to be very effective in inhibiting initial mineral phase formation, growth and demineralization of the preformed mineral phase of calcium phosphate (CaP), but also showed significant inhibition towards the growth of calcium oxalate ($CaOx$) crystals. To validate *in vitro* results thus obtained aqueous extract of *Tamarindus indica* was given to the hyperoxaluric rat models. Stone formation was induced in animals by giving 0.4% ethylene glycol and 1.0% ammonium chloride in their drinking water for 9 and 15 days. Experimentally induced hyperoxaluria in rats was treated with 5% and 10% aqueous extract of *Tamarindus indica* separately in two groups. Treated rats showed normalized levels of serum urea, serum creatinine, creatinine clearance and kidney injury marker enzymes, viz. alkaline phosphatase and lactate dehydrogenase, which were comparable to that of control group animals which were not given any treatment in 9 days and 15 days. Isolation and purification of potent biomolecule(s) was done by using techniques viz. dialysis, anion exchange chromatography and molecular sieve chromatography. A protein band of molecular weight of $\backsim$ 97 kDa was thus purified by SDS-PAGE possessing an ability to inhibit CaP mineralization as well as $CaOx$ crystal nucleation and growth.

Inhibitory potency of *Terminalia arjuna* was checked against calcium phosphate initial mineral phase formation, its subsequent growth and demineralization of preformed mineral phase and also against calcium oxalate crystals growth. Crude aqueous extract of *Terminalia arjuna* was then subjected to bioassay guided successive solvent extraction method. After this extraction method saponin rich n-butanol fraction was obtained which showed significant inhibition not only against initial mineral phase formation of CaP but also against $CaOx$ crystal growth. Further, *in vivo* effect of this butanol fraction

in ethylene glycol induced hyperoxaluric rat model was also investigated. This butanol fraction was then given to hyperoxaluria rats at a dose of 25 mg/kg body weight and 50 mg/kg body weight to two separate groups for treatment periods of 9 and 15 days. This saponin rich butanol fraction showed inhibition not only *in vitro* but *in vivo* as well. Urine and serum analysis was done before sacrificing the animals. Saponin rich n-butanol fraction of *Terminalia arjuna* bark showed improved physiology which was further supported by histopathological studies of kidney tissue of the animals. Purification and identification of potent biomolecule from *Terminalia arjuna* bark was carried with ultrafiltration of aqueous extract. Further, purification of protein molecule was done with anion exchange chromatography and molecular sieve chromatography. Extent of purity was checked by running SDS-PAGE and bands were visualized by performing silver staining. Inhibitory activity of the protein was checked against mineralization of calcium phosphate (CaP) along with nucleation and crystal growth of calcium oxalate ($CaOx$). Enhanced inhibition was found after each step of purification and a protein was purified having molecular $\backsim$ 14 kDa. Hence, both *Tamarindus indica* and *Terminalia arjuna* were found to be efficient in management of kidney stones not only *in vitro* but *in vivo* too.

Chapter 1

Introduction

Stone formation in the kidney is one of the oldest and most wide spread diseases known to man. Urinary calculi have been found in the tombs of Egyptian mummies dating back to 4000 BC [1] and in the graves of North American Indians from 1500 - 1000 BC [2]. Unfortunately, kidney stones are one of the most common disorders of the urinary tract. Each year, almost 3 million patients visit health care providers and more than half a million go to emergency rooms for problems related to kidney stones. Urolithiasis refers to the condition of having calculi in the urinary tract (which also includes the kidneys), which may form or pass into the urinary bladder. Nephrolithiasis refers to the condition of having kidney stones. Ureterolithiasis is the condition of having a calculus in the ureter, the tube connecting the kidneys and the bladder.

Kidney stones are small, hard lumps like stones that form in the kidneys. Kidneys clean blood by removing waste products and water to produce urine. Normally, the waste products in urine are present in very small amounts so they stay dissolved in the fluid. But sometimes they can become solid and form crystals on the inner surface of kidneys. Over time, these crystals may combine to

form a small and hard stone. These stones so formed are referred to as kidney stones. Kidney stone is a common chronic disorder affecting $10 - 15\%$ of the general population world wide. Calcium containing stones are the most common comprising about 75% of all urinary calculi, which may be in the form of pure calcium oxalate (50%) or calcium phosphate (5%) and a mixture of both (45%). Calcium oxalate stones are found in two different varieties, calcium oxalate monohydrate (COM) or Whewellite, and calcium oxalate dihydrate (COD) or Weddellite. COM is the most stable form among all calcium stones. Its frequency of occurrence in the clinical stones is much greater than COD and it has a high affinity for tubular cells of renal tissue.

In India, 12% of the population is expected to have urinary stones, out of which 50% may end up with loss of kidneys or renal damage [3]. Thus, the disease is as widespread as it is old, particularly in countries with dry and hot climate. "Stone belt" regions of the world are located in countries of Middle East, North Africa, the Mediterranean Regions, North Western state of India and Southern State of USA and areas around the great lakes. In India, the "stone belt" occupies parts of Maharashtra, Gujarat, Punjab, Haryana, Delhi and Rajasthan. In these regions, the disease is so prevalent that most of the members in a family suffer from kidney stones at some point in their lives. In United States, patients with kidney stones have been increasing over the past 30 years. In the late 1970s, less than 4 percent of the population had stone forming disease. By the early 1990s, the portion of the population with the disease had increased to more than 5 percent. Caucasians are more prone to develop kidney stones than African Americans.

Biomineralization is the process by which living organisms produce minerals, often to harden or stiffen existing tissues. It is an extremely widespread phenomenon which is used by all six taxonomic kingdoms to form minerals [4].

Five examples of biomineralization are:

1. The formation of siliceous spicules and frustules in sponges and diatoms, respectively.
2. The structure of skeletal spicules composed of amorphous calcium carbonate in some tunicates.
3. The secretion of the prism and nacre of some molluscan shells.
4. The development of skeletal spicules of sea urchin embryos.
5. The formation of bones and enamel of teeth in vertebrates.

The skeleton and teeth are normally the only mineralized tissues or organs in the human body. Such mineralization takes place under controlled mineral balance in the body. Any imbalance of minerals in the body may lead to some pathological condition. Such a condition where inappropriate biomineralization takes place in soft tissues, is called as ectopic calcifications. Ectopic calcifications are typically composed of calcium phosphate salts, including hydroxyapatite, but can also consist of calcium oxalates and octacalcium phosphate as seen in kidney stones [5]. In uremic patients, a systemic mineral imbalance is associated with widespread ectopic calcification, referred to as metastatic calcification [6]. In the absence of a systemic mineral imbalance, ectopic calcification is typically termed as dystrophic calcification, where blood levels of calcium are normal, and abnormalities or degeneration of tissues result in mineral deposition. Metastatic calcification can occur widely throughout the body but principally affects the interstitial tissues of the vasculature, kidneys, lungs, and gastric mucosa. For the later three, acid secretions or rapid changes in pH levels contribute to the formation of salts. Increased level of the salts in urine leads to the supersaturation of urine and increased salt, usually calcium, gives rise to hypercalciuria. Hypercalciuria is the most common metabolic abnormality observed in patients with

nephrolithiasis. Hypercalciuria raises urine supersaturation with respect to the solid phases of calcium oxalate and calcium phosphate, leading to an enhanced probability for nucleation and growth of crystals into clinically significant stones [7].

About 90% of stones are 4 mm or less in size and usually pass spontaneously. However, 9% of stones, larger than 6 mm require some form of intervention. There are various measures which are used to encourage the passage of stones. These include increased hydration, medication for treating infection and reducing pain, and diuretics to encourage urine flow and prevent further stone formation. Cautions are usually taken in eating certain foods with high concentrations of oxalate which may precipitate and lead to acute renal failure in patients with chronic renal disease [8]. Surgery is necessary when pain is persistent and severe in renal failure and when there is a kidney infection. It is also advisable if the stone fails to pass or move after 30 days of other treatments. Finding a significant stone before it passes into the ureter allows physicians to fragment it surgically before it causes any severe problems. In most of these cases, non-invasive extracorporeal shock wave lithotripsy (ESWL) is used. Percutaneous nephrolithotomy (PCNL) or rarely open surgery may ultimately be necessary for large or complicated stones or stones which fail other less invasive attempts at treatment. A single retrospective study in the USA, at the Mayo Clinic, has suggested that lithotripsy may increase subsequent incidence of diabetes and hypertension [9]. More common complications related to ESWL are bleeding, pain related to passage of stone fragments, failure to fragment the stone and the possible requirement for additional or alternative interventions. Phytotherapy is the alternative to avoid complications arising due to medical treatments. It is well documented in Indian Ayurvedic system and relies on the use of plants, either whole or in the form of prepared extracts and essences.

For thousands of years, plants were a primary source of therapeutic medication for cultures all over the world. The European Scientific Cooperative on Phytotherapy (ESCOP), established in 1989, defines phytomedicines as "medicinal products containing as active ingredients only in plants, parts of plants or plant materials, or combinations thereof, whether in the crude or processed state. Plant materials include juices, gums, fixed oils, essential oils, and any other directly derived crude plant product. They do not include chemically defined isolated constituents, either alone or in combination with plant materials" (European Society Cooperative on Phytotherapy, n.d.). ESCOP supports clinical studies on the safety and efficacy of phytotherapeutic agents. The primary role of phytotherapy in the problem of kidney stones is to use plants with the following purposes:

- Antispasmodic and sedative plants to reduce pain caused by renal colic.
- Diuretic plants to increase urination, and permit the removal of the stones.
- Bactericide plants to prevent infections.
- Plants that prevent the formation of kidney stones.

The marketed composite herbal formulations, Cystone (Himalaya Drug Company, India), Calcuri (Charak Pharmaceuticals, Bombay, India) and Chandraprabha bati (Baidyanath, India) have been widely used clinically to dissolve urinary calculi in the kidney and urinary bladder [10].

Nearly, 15% of the population of northern India suffers from kidney stones [3]. However, fewer occurrences of urinary calculi are found in southern India, which may be due to regular dietary intake of tamarind [3]. Imli or *Tamarindus indica* also called Indian Date, is a large, broad-leaved, tropical tree found in India and large parts of Asia. It is routinely used by practitioners of Ayurvedic system of medicine in the treatment of urinary stone disease. Many plants have

been reported all over the world which are able to inhibit kidney stones. Interestingly, the consumption of some of these plant products is also very high in areas where the incidence of the disease is reported to be very low. *Tamarindus indica* belongs to the Dicotyledonous family Leguminosae. Another plant, *Terminalia arjuna*, belongs to family Combretaceae. It is a large tree distributed throughout India. It is a commonly occurring medicinal plant growing as a 20-30 m high tree. In India, plant is found in plenty throughout in sub Himalayan tracts of Uttar Pradesh, South Bihar, Madhya Pradesh, Delhi, Deccan region mainly along riverside, riverlets and ponds. The bark of *Terminalia arjuna* is known for treating heart diseases, coronary artery diseases and hypercholesterolemia [11]. Aqueous extract of *Terminalia arjuna* bark is shown to protect the liver and kidney tissues against CCl_4- induced oxidative stress probably by increasing antioxidative defense activities. Its aqueous extract prevents carbon tetrachloride induced hepatic and renal disorders [12]. Keeping in mind the complications arising due to the surgical treatment of kidney stones and medicinal value of *Tamarindus indica* and *Terminalia arjuna* in this context, the scientific basis of their antilithiatic properties have been investigated using both *in vitro* and *in vivo* methods with the following objectives:

1. To study the effect of aqueous extract of *Tamarindus indica* and *Terminalia arjuna* on the extent of precipitation of calcium and phosphate (CaP) and calcium and oxalate ($CaOx$) using the homogenous system of *in vitro* mineral phase formation.

2. To study the effect of aqueous extract of *Tamarindus indica* and *Terminalia arjuna* on the growth and demineralization of the preformed mineral phase using the homogenous system *in vitro*.

3. To isolate, purify and characterize the new biologically active compounds from aqueous extract of *Tamarindus indica* and *Terminalia arjuna* which

have the ability to influence mineralization and demineralization reactions.

4. To investigate the effect of *Tamarindus indica* and *Terminalia arjuna* on experimentally induced hyperoxaluria and nephrocalcinosis in rats.

Chapter 2

Review of literature

Kidney stone formation or urolithiasis is a complex process that results from a succession of several physicochemical events including supersaturation, nucleation, growth, aggregation, and retention within the kidneys. Epidemiological data have shown that calcium oxalate is the predominant mineral in a majority of kidney stones. Kidney stones have afflicted many famous historical figures, including Benjamin Franklin, Isaac Newton, Peter the Great and Louis XIV. X-rays of Egyptian mummies dating back eight thousand to ten thousand years ago show evidence of stones. Caesar Augustus, the first emperor of the Roman Empire, suffered from various physical maladies including kidney and bladder stones.

2.1 Epidemiology of kidney stones

Urolithiasis is a longstanding medical illness and still a common public health problem. It affects up to 20% of the general population worldwide [13]. In the United States, up to 12% of men and 6% of women will develop a renal stone at some point in life [14]. While in Middle Eastern countries, the lifetime

prevalence of kidney stone is even higher [15]. In India, 12% of the population is expected to have urinary stones, out of which 50% may end up with loss of kidneys or renal damage. Also, nearly 15% of the population of northern India suffers from kidney stones [3] Recurrence rates as high as 50% in 10 years have also been documented [16].

2.2 Composition of urinary calculi

Calcareous stone is the most common type of kidney stone disease. It accounts for upto 80% of all stones [17]. The primary chemical complexes are calcium oxalate ($CaOx$) and calcium phosphate (CaP). Uric acid (UA) stone represents about $10-15\%$ and the other less frequent types of kidney stones are magnesium ammonium phosphate (MAP) or struvite stones, ammonium urate stones, cystine stones, xanthine and other miscellaneous stones (Table) There are four main types of kidney stone, each with different causes.

2.2.1 Calcium stones

Calcium stones are formed from calcium and phosphate, or calcium and oxalate. About $70-80\%$ of kidney stones are calcium stones. They usually develop in high levels of calcium in the urine. High levels of calcium in your urine can be caused by:

- Some cancers and kidney diseases
- Sarcoidosis (a condition causing inflammation of the lymph nodes and other organs)
- Treatment with thyroid hormones or some diuretics
- Intestinal bypass surgery

- Genetic factors
- Eating a diet heavy in meat, fish and poultry
- Taking large amounts of vitamin D. High levels of calcium in the urine can be due to parathyroid glands (glands which control how much calcium is in body) are overactive.

2.2.2 Struvite stones

Struvite stones contain magnesium and the waste product ammonia, and are almost always formed after long-term urinary tract infections. Struvite stones are usually large and are usually found in women.

2.2.3 Uric acid stones

Uric acid stones are formed when there is too much acid in urine. They are likely to develop if one had chemotherapy. There are certain conditions which affect the body's ability to break down chemicals (such as gout), eating a high-protein diet that includes a lot of meat, or some genetic factors that make the condition more likely. About 10% of kidney stones are uric acid stones.

2.2.4 Cystine stones

Cystine stones are rare and are caused by a hereditary condition called cystinuria, which makes the kidneys create unusually high levels of certain chemicals. About 2% of kidney stones are cystine stones.

2.3 Etiology of Renal Calculus

Kidney stone form when there is a decrease in urine volume or an excess of stone forming substances in the urine. Dehydration through reduce fluid intake

Composition of Urinary Calculi

Composition	Percentage of All Calculi	Common Causes
Calcium oxalate	70	Hypercalciuria Hyperparathyroidism Hypocitruria Renal tubular acidosis
Calcium phosphate	15	Hypercalciuria Hyperparathyroidism Hypocitruria Renal tubular acidosis
Cystine	2	Cystinuria
Magnesium ammonium phosphate (struvite)	3	UTI caused by urea-splitting bacteria
Uric acid	10	Hyperuricosuria Increased urine acidity

Table 2.1: Composition of Urinary calculi.

or strenuous exercise without adequate fluid replacement increases the risk of kidney stones. Obstruction to the flow of urine can also lead to stone formation. A number of different conditions can lead to kidney stones. Kidney stones may occur due to underlying metabolic conditions such as renal tubular acidosis [17] in which the kidneys are unable to excrete acids, Dent's diease, a rare X-linked recessive inherited condition which leads to kidney diseases [18], Primary hyperoxaluria [19], gout or hyperuricemia [20]. Other conditions associated with an increased risk of kidney stone include genetics, age and sex. Nanobacteria have also been found responsible for formation of kidney stones [21].

2.4 Major theories proposed to explain stone formation and growth

1. Supersaturation: This theory is based on the binding of salts, which occurs after a certain concentration is obtained [7]. If the salt concentration is less, the compound remains in solution. However if salt concentration exceeds, the compound precipitates. Temperature and the pH of a solution also affect solubility.

2. Crystallization or Nucleation: The crystallization or nucleation theory states that when ions or molecules in a dissociated state bind, crystals form. These crystals cluster to form lattice structures. Crystals are nucleated and grow by aggregation. There have been many studies on the possible mechanisms of crystal aggregate formation following the initial nucleation of crystals which is referred to as nucleation theory [22].

3. Reduction of inhibitors: The Inhibitors deficiency theory Inhibitors are substances that modify or alter crystal growth, thus preventing stone formation. Although urine may be supersaturated with a salt, these in-

hibitors can prevent stone formation. These molecules work by forming complexes with active surface compounds, which reduces their binding of calcium to oxdiate. Urinary inhibitors are involved in the control of these phenomena [23]. Citrate is the most important urinary stone inhibitor, Magnesium, pyrophosphate, nephrocalcin, glycosamine, RNA fragements are other important stone inhibitors. The absence or reduction of these inhibitors can aid in the production of stone formation.

2.5 Pathogenesis of Stones

Urinary stones or kidney stone formed when the normal balance of water, salt, minerals and other things found in the urine changes. On the one hand kidney must play an important role in water conservation, but at the same time, minerals with low solubility need to be excreted. In general renal stone are of different types, and each type of stone has its own group of causes, however, all four types of renal stones share a common pathogenesis that is based essentially upon excessive super saturation of the urine with poorly soluble material. Under supersaturated condition both homogenous and heterogeneous nucleation occurs. As a result crystal growth proceeds small crystals evolve into large crystals. Alternately many small crystals aggregate to form crystal aggregates (Figure 2.1).

Urine saturation can be increased by a deficiency of inhibitors of crystal growth, e.g. citrate, Magnesium, Pyrophosphate, glycosaminoglycans, by dehydration or over excretion of calcium. Tamm-Horesfall protein (THP), a mucoprotein secreted in the kidney, acts to reduce formation of aggregates of stone complexes and is found in lower amounts in chronic stone formers. Other studies have found that THP has a dual role as a modifier of crystal aggregation. In solutions with high pH and low concentrations of calcium and THP, THP acts as a

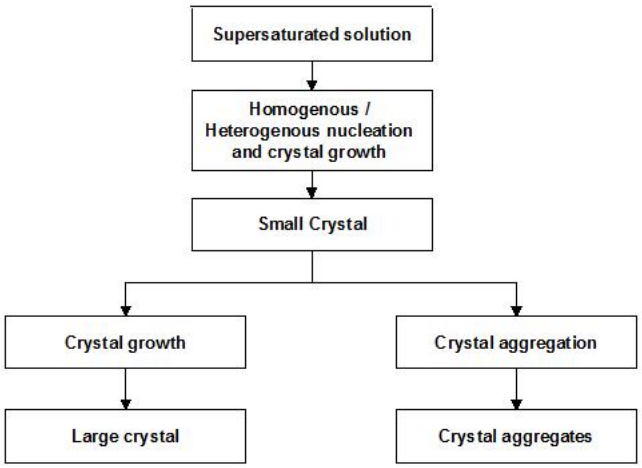

Figure 2.1: Stone formation

powerful inhibitor of calcium oxalate crystal aggregation. Conversely, solutions with low pH and high concentrations of calcium and THP, favor self-aggregation of THP molecules which lowers their inhibitory activity against calcium oxalate crystal aggregation [24].

Magnesium is usually deficient in stone formers as low magnesium intake has been linked to an increase in kidney stone formation by increasing the solubility of calcium oxalate stones. With an acute onset of kidney stones increasing the magnesium intake to 2 gm/d for 5 days followed by 500 mg twice a day helps to eliminate further stone formation and may act to decrease the size of an existing one. Magnesium should be given in higher doses on a continual basis for those patients who have had more than one episode of nephrolithiasis. Glycosaminoglycans and other semi-synthetic sulfated polysaccharides have been shown to impede urolithiasis by preventing crystal adherence, correction of abnormal oxalate flux, inhibition of crystal growth and agglomeration and prevention of renal tubule damage [25].

2.6 Diagnosis of kidney stones

The diagnosis of a kidney stone can be confirmed by radiological studies or ultrasound examination; urine tests and blood tests are also commonly performed. Clinical diagnosis is usually made on the basis of the location and severity of the pain, which is typically colicky in nature (comes and goes in spasmodic waves). Pain in the back occurs when calculi produce an obstruction in the kidney. Imaging is used to confirm the diagnosis and a number of other tests can be undertaken to help establish both the possible cause and consequences of the stone.

2.6.1 X-rays

The relatively dense calcium renders these stones radio-opaque and they can be detected by a traditional X-ray of the abdomen that includes the Kidneys, Ureters and Bladder - KUB [26]. This may be followed by an IVP (Intravenous Pyelogram) which requires about 50 ml of a special dye to be injected into the bloodstream that is excreted by the kidneys and by its density helps outline any stone on a repeated X-ray. These can also be detected by a Retrograde pyelogram where similar "dye" is injected directly into the ureteral opening in the bladder by a surgeon, usually a urologist. About 10% of stones do not have enough calcium to be seen on standard x-rays (radiolucent stones).

2.6.2 Computed tomography

Computed tomography without contrast is considered the gold-standard diagnostic test for the detection of kidney stones. All stones are detectable by CT except very rare stones composed of certain drug residues in the urine [26]. If positive for stones, a single standard x-ray of the abdomen (KUB) is recommended. This gives a clearer idea of the exact size and shape of the stone as

well as its surgical orientation. Further, it makes it simple to follow the progress of the stone by doing another x-ray in the future. Drawback of CT scans include radiation exposure and cost.

2.6.3 Ultrasound

Ultrasound imaging is useful as it gives details about the presence of hydronephrosis (swelling of the kidney - suggesting the stone is blocking the outflow of urine) [26]. It can also be used to detect stones during pregnancy when x-rays or CT are discouraged. Radiolucent stones may show up on ultrasound however they are also typically seen on CT scans. However, it is sometimes recommend that US be used as the primary diagnostic technique with CT being reserved for those with negative US result and continued suspicion of a kidney stone. This is due to its lesser cost and avoidance of radiation.

2.6.4 Other

In addition to the techniques mentioned, there are some other investigations also which are performed in order to confirm the presence of the stones in the body. These investigations include:

- Microscopic study of urine, which may show proteins, red blood cells, bacteria, cellular casts and crystals.
- Culture of a urine sample to exclude urine
- Blood tests: Full blood count for the presence of a raised white cell count (Neutrophilia) suggestive of infection, a check of renal function and to look for abnormally high blood calcium blood levels (hypercalcaemia).
- 24 hour urine collection to measure total daily urinary volume, magnesium, sodium, uric acid, calcium, citrate, oxalate and phosphate.

2.7 Treatment of kidney stones

2.7.1 Renal calculi

The characteristics of the stones (size, number, location, and composition), renal anatomy, and clinical factors are all considered when selecting a treatment approach for renal calculi.

2.7.2 Simple renal calculi

Simple renal calculi are those with a stone burden of < 2 cm (aggregate diameter) and normal renal anatomy. Most simple renal calculi ($80 - 85\%$) can be treated successfully with shock wave lithotripsy [27]. However, lithotripsy may fail or be less effective when stones are larger; stones are located in dependent or obstructed parts of the collecting system; stones are made up of calcium oxalate monohydrate, brushite, or cystine; the patient is obese or has a body build that inhibits proper imaging; or it is difficult to target the stone for shock wave delivery and subsequent fragmen-tation [28].

A retrospective comparison of percutaneous nephrolithotomy and shock wave lithotripsy found that as stone burden increased, the number of lithotripsy treatments and ancillary procedures increased, but stone-free rates decreased [29, 30]. Percutaneous nephrolithotomy results in higher stone-free rates and lower retreatment rates than shock wave lithotripsy. Because it is more invasive, however, percutaneous nephrolithotomy is usually reserved for patients in whom shock wave lithotripsy fails or those who are unsuitable for lithotripsy. Ureteroscopy is an increasingly used alternative for treating simple renal calculi because it has similar stone-free rates to shock wave lithotripsy and morbidity is lower than with per-cutaneous nephrolithotomy. Ureteroscopy is especially attractive in coagulopathic, pregnant, or morbidly obese patients where

shock wave lithotripsy or percutaneous nephrolithotomy are less effective or contraindicated.

2.7.3 Complex renal calculi

Complex renal calculi include stones > 2 cm, such as staghorn calculi; stones occurring in kidneys with abnormal anatomy; and stones resistant to fragmentation. Recently published guidelines of the American Urologic Association recommend that staghorn calculi should not be treated with lithotripsy because of relatively poor stone-free rates. Ureteroscopy has been used to treat upper tract stones > 2 cm, but stone clearance rates are significantly lower than with percuta-neous nephrolithotomy and stones recur rapidly (16% within six months) [31].

For this reason, percutaneous nephrolithotomy is the treatment of choice for most complex renal stones. Combined percutane-ous nephrolithotomy and shock wave lithotripsy (sand-wich therapy) for complex stones was commonplace in the 1990s, but improvements in percutaneous neph-rolithotomy techniques have led to a decline in the need for shock wave lithotripsy [32]. Even the largest staghorn calculi can be cleared percutaneously with the aid of secondary look nephroscopy and multiple access tracts.

The management of lower pole calyceal calculi remains controversial. A prospective randomised multi-centre trial showed that percutaneous nephrolithotomy was better than shock wave lithotripsy in the clearance of lower pole calculi > 1 cm (stone-free rates of 91% v 21%). However, for lower pole calculi < 1 cm, a recent prospective randomised trial failed to show a statistically significant difference in stone-free rates between the two techniques. Urolithiasis associated with aberrant renal anatomy can present a treatment challenge. All three techniques described above and even laparoscopy have been used to treat calculi

in these situations.

2.7.4 Ureteral calculi

Ureteral calculi most commonly present with symptoms of acute renal colic. If urgent intervention is not needed the patient and clinician must decide whether to intervene or proceed with expectant management. The likelihood of spontaneous passage decreases as stone size increases. An extensive meta analysis found that most ureteral calculi < 5 mm in diameter pass through the urinary tract spontaneously [33]. Spontaneous passage usually occurs within four weeks after the onset of symptoms [33]. If a stone has not been passed within four weeks, intervention is indicated, as the risk of complications such as ureteral strcture and renal deterioration increase. Therefore, observation is adequate for stones < 5 mm if symptoms can be controlled and follow-up is ensured. For the purposes of selecting treatment, ureteral calculi can be divided into categories on the basis of location.

2.7.4.1 Proximal ureteral calculi

Several endourological options are available for the treatment of proximal ureteral stones: shock wave lithotripsy with or without stone manipulation, ureteroscopy, and percutaneous nephrolithotomy. In 1997, the ureteral stones guidelines panel of the American Urologic Association recommended shock wave lithotripsy as the treatment of choice for stones in the proximal ureter, with stone-free rates up to 85%. A retrospective series noted that proximal ureteral stones > 1 cm have poor stone-free rates with this treatment [34]. However, flexible ureteroscopy is increasingly popular as primary treatment for proximal ureteral stones as a result of the availability of small diameter flexible ureteroscopes, ureteral access sheaths, holmium laser lithotripsy, and stone baskets. Percutaneous

nephrolithotomy is reserved for large or impacted proximal ureteral stones.

2.7.4.2 Distal ureteral calculi

Although the likelihood of spontaneous passage of stones is highest in the distal ureter, intervention with ureteroscopy or shock wave lithotripsy is often necessary. Both techniques are excellent options for symptomatic ureteral calculi < 1 cm. Randomised controlled trials comparing the two techniques have reached conflicting conclusions [35]. Unlike shock wave lithotripsy, ureteroscopy is not influenced by stone size and can be used to treat distal ureteral calculi > 1 cm. Semirigid ureteroscopy has a success rate of $90 - 99\%$ for treating distal ureteral stones. Ureteroscopy may also be the simplest solution in institutions with limited access to a lithotripter.

2.8 Prevention and management of kidney stones

There are many preventive strategies available for kidney stones. Main strategies include improved dietary habits. A diet low in protein, nitrogen and sodium is usually advised. Adequate levels of calcium and oxalate are also taken care of. Oxalate-rich foods, such as chocolate, nuts, soybeans, rhubarb and spinach are avoided [36]. However, an adequate intake of dietary calcium is also advised. In case of formation of kidney stones, drugs such as thiazides, potassium citrate, magnesium citrate and allopurinol are taken depending on the cause of stone formation.

Most kidney stones do not require surgery and pass on their own. Surgery is necessary when the pain is persistent and severe, in renal failure and when there is a kidney infection. Most simple renal calculi $(80 - 85\%)$ can be treated successfully with shock wave lithotripsy [27]. However, lithotripsy may fail or be less effective when stones are larger; stones are located in dependent or ob-

structed parts of the collecting system; stones are made up of calcium oxalate monohydrate, brushite, or cystine; the patient is obese or has a body build that inhibits proper imaging; or it is difficult to target the stone for shock wave delivery and subsequent fragmentation [29, 30, 28]. A retrospective comparison of percutaneous nephrolithotomy and shock wave lithotripsy found that as stone burden increased, the number of lithotripsy treatments and ancillary procedures increased, but stone-free rates decreased [27, 30, 29]. Percutaneous nephrolithotomy results in higher stone-free rates and lower retreatment rates than shock wave lithotripsy [27]. However, in case of coagulopathic, pregnant, or morbidly obese patients shock wave lithotripsy or percutaneous nephrolithotomy are less effective or contraindicated. Further, a single retrospective study in the USA, at the Mayo Clinic, has suggested that lithotripsy may increase subsequent incidence of diabetes and hypertension [9] (Figure 2.2).

2.8.1 Expulsive theory

This treatment comprises the use of drugs to help the spontaneous passage of ureteral calculi. Several drugs including calcium channel blockers (nifedipine), steroids, and α adrenergic blockers have recently been investigated [34, 35]. The rationale for using α blockers is based on the presence of large numbers of $\alpha 1$ adrenoceptors in the distal ureter. These blockers inhibit basal ureteral tone and peristaltic frequency and decrease the intensity of ureteral contractions.

2.8.2 Other treatments

Certainly and increased oral fluid administration is in order unless there is a blockage of the ureter or kidney. Then, surgical, lazer or shock wave intervention is in order to dislodge the stone. For the passage of smaller stones, hot packs over the affected flank helps to relax tense muscles form pain and spasm, allowing

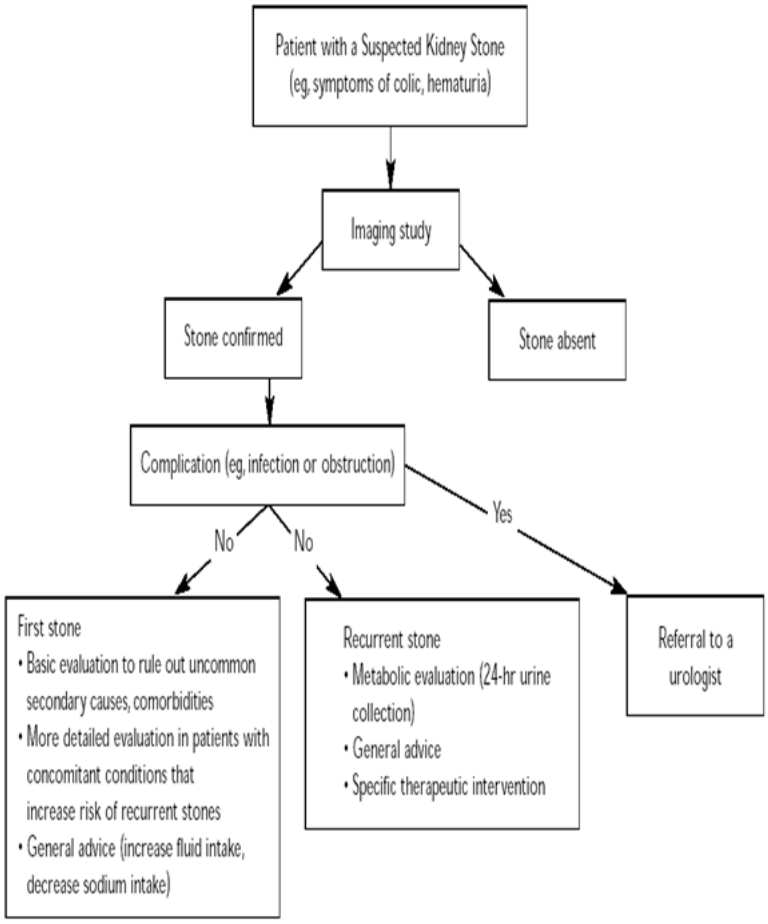

Figure 2.2: Evaluation and management of a patient with kidney stones.

easier passage of the stone. These can be left in place for considerable periods as long as they are wet packs and are not extracting moisture from the skin. Caution should be taken in the elderly and diabetics as they are less sensitive to heat and burning of the skin may ensue. Increased fluid intake helps pass most stones which are 5 mm or less in size. An initial abdominal x-ray is obtained in order to locate the stone and measure its size. This helps in making management decisions initially as larger stones may need surgical or ESWL therapy.

ESWL therapy is most effective with stones measuring 5 mm up to 2 cm but begins to loose its effectiveness the larger they become. Struvite stones, depending upon the location and size may need surgical intervention, especially if they are causing renal impairment. While ESWL has had some success in eliminating stones there has been some concern that it has limitations in the pediatric population and that its role needs to be redefined. In a study examining ESWL's role in pediatric nephrolithiasis, surgery was ultimately needed in a large number of cases following treatment[37]. Despite the widespread clinical use of ESWL, the margin of safety for the kidney during shock wave application is largely unknown. In a study done on rabbits, dose-dependent moderate damage (subcapsular hemorrhage, interstitial hemorrhage, capsular tension and perirenal hemorrhage) were noted in all kidneys at 24 hours following treatment. Evidence of permanent changes (some fibrosis, tubular and glomerular damage, chronic inflammatory alterations) was noted in long-term follow up, while a complete necrosis of the treated kidney was not encountered [38].

While ESWL is often the first choice of therapy its limitations are also well established: silent calyceal stones, calyceal diverticula stones, nephrolithiasis in horse-shoe kidneys, medullary sponge kidney, and residual fragments after ESWL. Other methods and further refinement of ESWL are currently being examined to decrease morbidity and the need for further invasive treatments.

For smaller stones less than 5 mm, medical management is usually all that is necessary. Increasing the fluid intake and alkalizing the urine, if it is acidic, or acidifying it if it is alkaline, help to make the stone more soluble and easier to pass. Demulcents such as Althea or Ulmus, coupled with a mild diuretic such as Galium or Zea mays also are useful. An anti infective such as Uva ursi or Barosma added also help to decrease the risk of infection. In cases where there is severe colic from passage of the stone certain homeopathic preparations can help in alleviating the pain and with passage. Dioscorea administered in drop doses along with Piscidia and Belladonna will help with pain relief. Hot packs to the affected flank is also useful for pain relief and ureteral dilation.

2.9 Phytotherapy

Phytotherapy is the study of the use of extracts from natural origin as medicines or health-promoting agents. Although regarded as "alternative medicine" by much of Western medicine, phytotherapy, when critically carried out, is an important field of pharmacognosy Out of the total 4, 22, 000 flowering plants reported from the world [39], more than 50,000 are used for medicinal purposes. In India, more than 43% of the total flowering plants are reported to be of medicinal importance. Utilization of plants for medicinal purposes in India has been documented long back in ancient literature [40]. However, organized studies in this direction were initiated in 1956 [41] and off late such studies are gaining recognition and popularity due to loss of traditional knowledge and declining plant population. Right from its beginning, the documentation of traditional knowledge especially on the medicinal uses of plants, has provided many important drugs of modern day [42, 43]. Even today this area holds much more hidden treasure as almost 80% of the human population in developing countries is dependent on plant resources for healthcare [44].

2.9.1 Herbal treatment of kidney stones

Herbal medicines have long played a role in the treatment and relief of nephrolithiasis. Used singly or in combination they act to increase diuresis, relieve pain and relax muscle spasm which often accompanies passage. While many herbal medicines affect the formation of kidney stones in some way, only a few exhibit a primary action for the condition. The beneficial effects caused by many herbal infusions on urolithiasis can be attributed to a disinfectant action as well as to the presence of saponins. Some solvent action with respect to the disrupting the formation of uric stones is primarily due to their capacity to alkalinize the urine[45]. In herbal treatment of kidney stones, antilithics are used to "dissolve" the stones or aid their passing to guard against further retention. Diuretic action is also needed to increase the amount of fluid going through the kidneys and flush out the deposits.

Previous clinical studies have shown that herbal medicines and their concoctions could be used to inhibit calcium oxalate crystallization. However, the pharmacodynamics and *in vitro* effects of such medicines have not been established. Five Chinese herbal medicines were selected based on their usefulness in treating stones disease [46]. Phytotherapy can reduce the recurrence rate of stones. The use of plant products with claimed uses in the traditional systems of medicine assumes importance. An excellent account of the 'Pashanabheda' group of plants, claimed to be useful in the treatment of urinary stones is given by Narayana Swami and Ali [47] and Mukerjee *et al.* [48]. In India, in the Ayurvedic system ofmedicine, Pashanabheda is the Sanskrit term used for a group of plants with diuretic and antilithiatic activities (Pashana = stone; Bheda = break).

2.9.2 Plants and plant products with antilithiatic activity

The marketed composite herbal formulations, Cystone (Himalaya Drug Company, India), Calcuri (Charak Pharmaceuticals, Bombay, India) and Chandraprabha bati Baidyanath, India) have been widely used clinically to dissolve urinary calculi in the kidney and urinary bladder. Pharmacological and clinical studies carried out on a composite herbal formulation, Trinapanchamool consisting of five herbal drugs namely *Desmostachya bipinnata*, *Saccharum officinarum*, *Saccharum nunja*, *Saccharum spontaneum* and *Imperata cylindrica* was found to be effective both as prophylactic in preventing the formation and as curative in dissolving the preformed stones in albino rats. The antilithiasis activity of this formulation has been attributed to its diuretic activity [49].

A study was undertaken to evaluate the *in vitro* Antilithiatic activity of aqueous extract and sodium hydroxide extract of seeds of *Dolichos biflorus* as therapy for lithiasis. The ethanol extract of *Asparagus racemosus* was more active than the methanol and distill water extract of the same plant. The combination of the two plants was not as active as the individual extract [50]. The seeds of *Dolichos biflorus* and rhizomes of *Bergenia ligulata* were also tested for their *in vitro* antilithiatic and anticalcification activity by the homogenous precipitation method. The extracts were compared with an aqueous extract of cystone (a marketed preparation) for their activities. Also a combination of the extracts of the two plants was tested. Extracts of *Dolichus biflorus* showed activity almost equivalent to cystone while *Bergenia ligulata* showed less activity and the combination was not as active as the individual extracts [51]. *In vitro* studies in which calcium oxalate precipitation was induced by addition of 0.1 M sodium oxalate to unfiltered urine samples from Wistar rats and normal humans in absence and presence of *Phyllanthus niruri* extract (0.25 mg/ml), suggested that extract may interfere with early stages of stone formation [52].

Phyllanthus niruri has an inhibitory effect on crystal growth in rat model of urolithiasis induced by introduction of calcium oxalate seed in bladder of rats. The effect may be due to higher levels of glycosoaminoglycans incorpated into calculi [53].

Investigations on the effect of *Ammi visnaga* seeds on kidney stones revealed that the antilithiatic effect is mainly because of highly potent diuretic activity and amelioration of uraemia and hyperbilirubinemia by seeds of *Ammi visnaga* [54]. Cranberry juice has antilithogenic properties as its ingestion significantly and uniquely altered urinary risk factors causing decreased excretion of oxalate and phosphate while increase in citrate excretion was noted [55]. *C. peltata* root powder decreased urinary oxalate, calcium, serum potassium likewise increased serum magnesium levels [56]. The effect of ingestion of 3 and 10 g of tamarind pulp (*Tamarindus indicus*) was studied in normal subjects and in stone formers. Tamarind intake at the dose of 10 g showed significant beneficial effect in inhibiting spontaneous crystallization in both normal subjects and in stone formers [57].

Studies on the stem juice of *Musa paradisiaca* were found to be effective in dissolving the phosphate type of stones in albino rats induced by foreign body insertion method using zinc discs [58]. In another experimental study stem juice of Musa significantly reduced the incidence of oxalate urolithiasis by lowering the activity of the enzyme glycolic acid oxidase [59]. The stem juice of Musa reduced urinary oxalate, glycolic acid, glyoxylic acid and phosphorus excretion in hyperoxaluric rats. Grases and co-workers of Division of Urochemistry, Department of Chemistry, University of Balaeric Islands, Spain have studied the antilithiatic activity of *Zea mays*, *Rosa canina*, *Herniaria hirsuta* and *Agropyron repens* in rats. The antilithiatic activity of *Zea mays* has been assigned to its diuretic activity [60]. *Rosa canina* was found to have significant activity on cal-

cium oxalate urolithiasis as it decreased calciuria and increased citraturia [61]. The antilithiatic activity of *Herniaria hirsuta* has been assigned to increase in citraturia [62] where as *Agropyron repens* did not show any positive effects on the risk factors of urolithiasis. The effect of *H. hirsuta* on the adhesion of calcium oxalate monohydrate crystals to renal cells was studied which indicated that *H. hirsuta* altered crystal adhesion only under conditions of increased fluidity [63].

2.9.2.1 *Tamarindus indica*

Tamarindus indicia, belongs to the Dicotyledonous family Leguminosae Sub Family Caesalpiniaceae, which is the third largest family of flowering plants with a total of 727 genera and 19,327 species. *Tamarindus indica* is a tropical evergreen tree native to fertile areas throughout Africa and Southern Asia. It is widely cultivated as an ornamental tree and for its acidic fruits used in making drinks and a popular component of many decoctions used as health remedies. In Northern Nigeria, the fresh stem bark and fresh leaves are used as decoction mixed with potash for the treatment of stomach disorder, general body pain, jaundice, yellow fever and as blood tonic and skin cleanser (Table 2.2).

Tamarindus indica contain high levels of crude protein [64]. *Tamarindus indica* is also high in carbohydrate, which provides energy, rich in the minerals, potassium, phosphorus, calcium and magnesium. *Tamarindus indica* can also provide smaller amounts of iron and vitamin A. The fruit pulp is the richest source of tartaric acid $(8 - 18\%)$ and seeds are rich of valuable amino acid [65]. The plant has a great phytochemical significance. On literature survey it was revealed that a variety of secondary metabolites have been reported from tamarind. The leaf oil contains thirteen components among which linonene (24.4%) and benzyl benzoate (40.6%) were most predominant [66]. The volatile constituents of the fruit pulp were furan derivatives (44.4%) and carboxylic acid (33.3%) of the total volatiles [67]. The major fatty acids of seeds were palmitic

Kingdom	Plantae
Division	Magnoliophyta
Class	Magnoliopsida
Order	Fabales
Family	Fabaceae
Genus	*Tamarindus* L.
Species	*Tamarindus indica* L.

Table 2.2: Classification of *Tamarindus indica*

Figure 2.3: *Tamarindus indica*

acid, oleic acid, linoleic acid and eicosanoic acid. Seven hydrocarbons, â-amyrin, compesterol and â-sitosterol were found in the unsaponifiable matter of seeds. The mucilage and pectin, arabinose, xylose, galactose, glucose and uronic acid were also identified [68] (Figure 2.3).

Tamarindus indica is an important food resource for the Thai population. The flower and leaf are eaten as vegetables [69]. *Tamarindus indica* is a plant widely used in traditional medicine in Africa for the treatment of many diseases such as fever, dysentery, jaundice, gonococci and gastrointestinal disorders [70]. Pharmacological investigations on *Tamarindus indica* extracts reported them to have antibacterial, antifungal, cytotoxic [70], gastrointestinal [71]. Aqueous extract of seed reduces blood sugar level [72] showes hypolipidemic effect, reduces $14 - 17\%$ of plasma lipid, total lipid, cholesterol, lipoprotein and triglycerides [73, 74]. The seed coat extract has strong antioxidant property, used as additive to food, cosmeticpharmaceutical preparations [75]. The fruit also has antimicrobial and antibiotic activity [76].

Kingdom	Plantae
Division	Magnoliophyta
Class	Magnoliopsida
Order	Myrtales
Family	Combretaceae
Genus	Terminalia
Species	T. arjuna
Botanical name	Terminalia arjuna

Table 2.3: Classification of *Terminalia arjuna.*

2.9.2.2 *Terminalia arjuna*

Terminalia arjuna is a large tree distributed throughout India. It is a commonly occurring medicinal plant growing as a 20–30 m high tree. In india, plant is found in plenty throughout In sub Himalayan tracts of Uttar Pradesh, South Bihar, Madhya Pradesh, Delhi, Deccan region mainly along riverside, riverlets and ponds. According to Bhava Prakash, as per ayurvedic pharmacology, it is laghu (light) and ruksh (dry) in properties, it has two main tastes kashya (astringent) and tickta (bitter). The virya i.e. the potency of Arjuna is sheet (cool) and vipaka i.e. the post digestive effect of Arjuna is katu i.e. pungent. It is kapha and pitta pacifying, cardiac restorative, helpful in wounds, Tuberculosis and poisoning; good for obesity and urinary disorders (Table 2.3).

It is well recognized in Ayurveda for its various therapeutic values [77] medicinal values of the plants are also well documented in Unani medicine therapies

Figure 2.4: *Terminalia arjuna*

[78]. *Terminalia arjuna* is known for its various medicinal properties like tonic, anthelmintic, styptic and alexiteric [79]. The bark of *Terminalia arjuna* is known for treating heart diseases, coronary artery diseases and hypercholesterolemia [11]. The plant has considerable importance as timber and its tannin containing nuts [80]. Polyphenolic contents were reported from *Terminalia Arjuna* bark [81]. The bark powder of *Terminalia arjuna* has also been found to improve antioxidant status in the patients of coronary heart disease and these beneficial effects may be related to its high flavonide content [82](Figure 2.4).

It has been well documented that bark extract contains acids (arjunic acid, terminic acid), glycosides (argentine arjunosides I-IV), strong antioxidants (flavones, tannins, oligomeric proanthocyanidins), minerals etc, but not much is known about the specific biological activity of individual constituents of this plant. Few of the active compounds from *T. arjuna* bark have been isolated and shown to possess antimutagenic and anticarcinoginic activity [83, 84]. Arjunolic acid, a new triterpene isolated from the bark of *Terminalia arjuna* have been reported

to have antioxidant and cardioprotective activity [85].

Not much antilithiatic properties of *Tamarindus indica* and *Terminalia arjuna* have been explored yet. Thus it was tempting to know their role in management of urolithiasis.

Chapter 3

Materials and methods

3.1 Preparation of aqueous extracts

3.1.1 *Tamarindus indica*

Commercially available tamarind fruit (*Tamarindus indica*) was bought from the local market, identified and then authenticated by microscopic and physio-chemical data. Fruit part was then soaked in double-distilled water overnight at 4°C to give a concentration of 10% (w/v). The extract so obtained was filtered through muslin cloth and subjected to centrifugation at 10,000 rpm for 20 minutes at 4°C in a cold centrifuge. The supernatant thus obtained was dried using a rotary evaporator. The final dried sample was stored in labeled sterile bottles and kept at −20°C. The final dried sample was reconstituted in water to 1000 µg/ml at the time of experiment and was referred to as aqueous extract of *Tamarindus indica*.

3.1.2 *Terminalia arjuna*

The dried bark of *Terminalia arjuna* was obtained from "Natural Remedies Pvt. Ltd." at Bangalore, India. A collection of voucher specimen is available with the company. The dried bark of *Terminalia arjuna* was boiled in distilled water. The extract was then filtered using Whatman No. 1 filter paper and the filtrate was evaporated in vacuo and dried using a rotary evaporator at 60°C. The final dried sample was stored in labeled sterile bottles and kept at -20°C. The final dried sample was reconstituted in water to 1000 µg/ml at the time of experiment and was referred to as aqueous extract of *Terminalia arjuna*.

3.2 Calcium and phosphate determination

3.2.1 Homogenous assay system of initial mineral phase formation of calcium and phosphate

A 5 ml homogenous assay was used to determine the extent of formation of calcium (Ca^{2+}) and phosphate (HPO_4^{2-}) precipitates *in vitro*. This 5 ml system was prepared by adding 0.5 ml of KH_2PO_4(50 mM), 0.5 ml of $CaCl_2$ (50 mM), 2.5 ml of Tris buffer (210 mM $NaCl$ + 0.1 mM Tris HCl) and 1 ml distilled water. This system was centrifuged at 4500 rpm and precipitates so obtained were dissolved in 5 ml of 0.1 N HCl. This 5 ml system for mineralization, already standardized in our laboratory was used to study the extent of *in vitro* mineral phase formation in the absence of any matrix [86, 87]. The $Ca^{2+}$and HPO_4^{2-}ions were estimated by the methods of Trinder [88] and Gomori [89], respectively.

3.2.2 Determination of calcium

Method of Trinder was used to determine the calcium ions in the sample.

Principle

Calcium ions get precipitated as napthyl hydroxamate 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 an orange red color, intensity of which is measured as the amount of calcium ions present in the sample.

Reagents

- Calcium reagent: This reagent was prepared by mixing two components and the volume was raised to 1000 ml by adding distilled water. The resultant mixture was filtered and stored in dark reagent bottle. Two components were made as follows:

  1. 280 mg Napthylhydroxamic acid in 100 ml of (95 ml double distilled water + 5 ml etholamine + 2 gm tartaric acid)
  2. 9 gm $NaCl$ in 500 ml double distilled water

- Color reagent: 60 gm ferric nitrate dissolved in 500 ml of acidified distilled water (485 ml double distilled water + 15 ml concentrated HNO_3). The volume was raised to 1000 ml with double distilled water.

- EDTA: 2 gm EDTA was dissolved in 0.1 N $NaOH$.

- Working standard: 2 mM of calcium chloride.

Procedure

0.1 ml of sample was taken in a test tube. To this 2.5 ml of calcium reagent was added and incubated it at 37°C for 30 minutes. After incubation, centrifugation was done at $45,000$ rpm for 15 minutes. Precipitates so obtained were dissolved

in EDTA and boiled for 10 minutes at 100°C followed by addition of 3 ml of color reagent. Absorbance was taken at 450 nm.

Calculations

The following formula was used to calculate the concentration of calcium ions (mM):

$$\frac{\text{Absorbance of test}}{\text{Absorbance of standard}} \times \text{Concentration of standard}$$

3.2.3 Determination of phosphate

The phosphate ion concentration in the sample was determined by the method of Gomori.

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 by mixing 2.5% ammonium molybdate dissolved in double 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 double distilled water.
- Working standard: 2 mM of KH_2PO_4.

Procedure

0.1 ml of sample was taken in a test tube. To this 0.24 ml of molybdic acid was added and incubated it at room temperature for 10 minutes. After incubation,

sample was diluted by adding 3 ml of distilled water. To this 0.1 ml of metol reagent was added and was mixed properly. Kept at room temperature for 20–30 minutes and measured absorbance at 660 nm.

Calculations

The following formula was used to calculate the concentration of phosphate ions (mM):

$$\frac{\text{Absorbance of test}}{\text{Absorbance of standard}} \times \text{Concentration of standard}$$

3.2.4 Homogenous assay system of growth and demineralization of calcium phosphate mineral phase

For the growth, first 5 ml systems were prepared in the same way as prepared for mineral phase. The calcium phosphate (CaP) precipitates so obtained were then resuspended in the same assay system. This assay system was incubated at 37°C. Calcium and phosphate ions were then estimated on the precipitates obtained after dissolving in 0.1 N HCl. Concentration of these ions represented the growth of calcium and phosphate precipitates over preformed initial mineral phase. To check the demineralization, again 5 ml system was prepared and precipitates were obtained. To this precipitate, 2.5 ml of Tris buffer (210 mM $NaCl$ + 0.1 mM Tris HCl) and 1.5 ml distilled water was added. This system was incubated at 37°C for 30 minutes. After incubation, it was centrifuged at 4500 rpm for 15 minutes. Calcium and phosphate ions were then estimated in the supernatant obtained after centrifugation.

Calculations

The following formula was used to calculate the concentration of calcium and phosphate ions (mM) both for growth and demineralization:

$$\frac{\text{Absorbance of test}}{\text{Absorbance of standard}} \times \text{Concentration of standard}$$

3.2.4.1 *In vitro* effect of crude aqueous extract of *Tamarindus indica* and *Terminalia arjuna* on homogenous assay system of initial mineral phase formation of calcium and phosphate, its subsequent growth and demineralization of preformed initial mineral phase formation

In vitro effects of aqueous extracts of *Tamarindus indica* and *Terminalia arjuna* were studied on homogeneous system of initial mineral phase formation for calcium phosphate (CaP), its subsequent growth and demineralization by employing 5.0 ml system. To see the inhibitory activity of aqueous extract, volume of distilled water was subsequently reduced from 1.5 ml to 0.0 ml in 5 ml system with an increasing volume of the aqueous extract ranging from 0.2 ml to 1.5 ml. Parallel control was also run in which no plant extract was added. This system was centrifuged at 4500 rpm and precipitates so obtained were dissolved in 5 ml of 0.1 N HCl. Calcium and phosphate ion estimation was performed by methods of Trinder and Gomori, respectively. In case of blank, sample was replaced with distilled water.

For growth, volume of the aqueous extract used on preformed calcium and phosphate precipitates was 0.05 ml, 0.10 ml, 0.15 ml and 0.2 ml in case of *Tamarindus indica* and 0.2 ml to 1.5 ml in case of *Terminalia arjuna*. Again, system was centrifuged at 4500 rpm and precipitates so obtained were dissolved in 5 ml of 0.1 N HCl. Calcium and phosphate ion estimation was performed by

methods of Trinder and Gomori.

To check the effect of aqueous extract on demineralization, again 5 ml system was prepared having no extract added to that and precipitates were obtained. To these precipitates, 2.5 ml of Tris buffer and increased volumes of extract ranging from 0.2 ml to 1.5 ml with subsequently reduced volume of water was added and then centrifuged at 4500 rpm for 15 min. Calcium and phosphate were then estimated in supernatant obtained after centrifugation. Extent of inhibition was checked in terms of percentage inhibition of calcium and phosphate ions formed in case of mineral phase and growth and in terms of percentage release of calcium and phosphate ions in case of demineralization.

Calculations

Percentage inhibition or percentage release of ions was calculated as $[(C - T)/C] \times 100$, where T is the concentration of Ca^{2+} or HPO_4^{2-} ion of the precipitate formed in test having plant aqueous extract and C is the concentration of Ca^{2+} or HPO_4^{2-} ion of the precipitate formed in control system which had distilled water (Millipore) and no plant extract.

3.3 Calcium oxalate detemination

3.3.1 *In vitro* nucleation assay of calcium oxalate ($CaOx$)

The extent of nucleation of calcium oxalate crystals was checked by the method of Hennequin [90]. Absorbance of the crystals formed was measured at 620 nm. In this system, OD at 620 is directly proportional to the mass of crystals formed per unit volume. Solutions of calcium chloride and sodium oxalate were prepared at the final concentration of 3 mmol/L and 0.5 mmol/L, respectively, in a buffer containing Tris 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 calcium chloride solution was mixed with 3.3 ml of the aqueous extract at different concentrations. Crystallization was started by adding 33 ml of sodium oxalate solution. The final solution was magnetically stirred at 800 rpm using a PTFE-coated stirring bar. The temperature was maintained at 37°C. The absorbance of the solution was monitored at 620 nm after every 1 minute till a constant line was obtained. OD was measured, during which turbidity slope was measured. The slope of the linear portion of the curve was determined during the time interval when the correlation coefficient of the curve, r^2, exceeds 0.99. In case of control, no plant extract was added.

3.3.1.1 *In vitro* effect of crude aqueous extract of *Tamarindus indica* and *Terminalia arjuna* on nucleation of calcium oxalate crystals

To see the effect of *Tamarindus indica* and *Terminalia arjuna* on nucleation, Hennequin method [90] was used. Experiments were conducted by using 20 µl and 40 µl of crude aqueous extract.

Calculations

The percentage inhibition produced by the additives was calculated as $[1 - (Tsi/Tsc)] \times 100$, where Tsc was the turbidity slope of control and Tsi the turbidity slope in the presence of inhibitor which was crude aqueous extract of the plant.

3.3.2 *In vitro* calcium oxalate crystal growth assay

Inhibitory activity against calcium oxalate ($CaOx$) crystal growth was measured using the seeded, solution-depletion assay [91]. Briefly, an aqueous solution of

10 mM Tris HCl 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). $CaOx$ monohydrate 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 detected spectrophotometrically at λ214 nm.

3.3.2.1 Effect of crude aqueous extract of *Tamarindus indica* and *Terminalia arjuna* on growth of calcium oxalate crystals

To see the effect of crude aqueous extracts of *Tamarindus indica* and *Terminalia arjuna*, their respective extracts were added to 4 ml growth assay system separately [92]. Volumes used were 10 µl, 20 µl, 40 µl, 60 µl and 80 µl. Rate of reduction of free oxalate was calculated using the baseline value and the value after 30 sec incubation with or without the extract. The relative inhibitory activity was calculated as follows: %Relative inhibitor activity $= [(C - S)/C] \times 100$, where C is the rate of reduction of free oxalate without any extract and S is the rate of reduction of free oxalate with plant extract.

3.4 Separation of biomolecules on the basis of molecular weight present in the crude aqueous extract

Purification of the potent biomolecule(s) from aqueous extract of *Tamarindus indica* and *Terminalia arjuna* having the ability to influence CaP and $CaOx$ mineralization reactions was started with the ultrafilteration of the crude aqueous extract of the plant by using Millipore ultrafilteration assembly having cut-

off molecular weight filters of 10 kDa. Effect of both fractions, having molecular weight less than 10 kDa and greater than 10 kDa along with the crude extract, was checked on CaP crystal mineralization along with $CaOx$ nucleation and growth. Further purification was carried out by using fraction which showed higher inhibitory activity having molecular weight more than 10 kDa.

3.5 Protein purification

3.5.1 Anion exchange chromatography

Principle

The separation of proteins in ion exchange chromatography depends on the reversible adsorption of charged solute molecules to immobilized ion exchange groups of opposite charge. To optimize binding of all charged molecules, the mobile phase is generally a low to medium conductivity (salt concentration) solution. The adsorption of the molecules to the solid support is driven by the ionic interaction between the two moieties and binding capacities are generally quite high. The strength of the interaction is determined by the number and location of the charges on the molecule and solid support. By increasing the salt concentration, the molecules with the weakest ionic interactions are disrupted first and elute earlier in the salt gradient. Those molecules that have a very strong ionic interaction require a higher salt concentration and elute later in the gradient.

Procedure

Preparation of column Before packing the column with the Macro - Prep® 25Q strong anion exchanger, the exchanger was washed with $2-3$ bed volumes of deionized water. After washing, exchanger was equilibrated with

Time (min)	Flow rate (ml/min)	Buffer
0-20	1.0	Buffer A
20-60	1.0	0-90% gradient of buffer A to buffer B
60-80	1.0	Buffer B
80-150	1.0	Buffer A

Table 3.1: Method used for anion exchange chromatography.

buffer A (20 mM Tris HCl + 0.1 M $NaCl$) having pH 7.4.

Sample loading and separation of biomolecules Plant fractions having molecular weight greater than 10 kDa were loaded in column (20×1.0 cm) packed with pre equilibrated Macro- Prep® 25Q strong anion exchanger (Bio-Rad laboratories) to separate the proteins present in the sample on the basis of their charge at a flow rate of 1 ml/minute. Bound proteins were eluted by incorporating a linear concentration gradient of $NaCl$ ($0.1-1$ M) in the column buffer while keeping the pH constant. All fractions were monitored for protein content (A_{280}) and simultaneously their conductivity was measured using Bio-Logic LP system. The fractions obtained after anion exchange chromatography were pooled and checked for their inhibitory activity against mineralization of CaP, nucleation of $CaOx$ and against growth of $CaOx$ crystals (Table 3.1).

3.5.2 Molecular sieve chromatography

Principle

It is a technique in which the material to be fractionated separates primarily according to molecular size or hydrodynamic radii. As it moves through a column of a neutral matrix and is washed with a solvent, molecules with larger radii elute out first followed by molecules having smaller radii.

Procedure

Column packing For molecular sieve chromatography, column packing was done with Bio-Gel P - 100 (Medium). In order to pack the column, 10 gm Bio-Gel P-100 (Medium) was added to degassed 20 mM Tris HCl buffer and was kept overnight at 20°C. After hydration supernatant was decanted. Washing was repeated for $2-3$ times with degassed buffer. Washed gel was packed in column (20×1.5 cm).

Sample loading and separation of biomolecules Most potent fraction obtained after anion exchange chromatography was lyophilized and dissolved in 1.5 ml of 20 mM Tris HCl buffer and was loaded in molecular sieve chromatography column as sample for further purification. Fractions were collected at a flow rate of 0.1 ml/min. Each fraction size was set at 0.5 ml. Fractions under all peaks were pooled and their inhibitory potency was again checked. Most potent peak was then loaded in 10% polyacrylamide gel. Bands were visualized after performing silver staining.

3.5.3 Electrophoresis

Principle

SDS – PAGE (Sodium Dodecyl Sulphate Poly Acrylamide Gel Electrophoresis) is a technique to separate proteins on the basis of their molecular weight. It uses an anionic detergent (SDS) to denature proteins. The protein molecules become linearized. One SDS molecule binds to 2 amino acids. Due to this, the charge to mass ratio of all the denatured proteins in the mixture becomes constant. These protein molecules move in the gel (towards the anode) on the basis of their molecular weights only and are separated.

Procedure

Most potent fractions obtained after molecular sieve chromatography for both the plants were loaded in one - dimensional SDS - PAGE to check the extent of purity. 10% separating and 4.4% stacking gels were made and run in Mini-Protein III apparatus (Bio-Rad Laboratories). Silver staining was done to visualize the protein bands by using ProteoSilver^{TM} Plus Silver Stain Kit (PROTSIL2, Sigma- Aldrich Co.).

3.6 Non protein purification of *Terminalia arjuna*

3.6.1 Bioactivity guided Successive Solvent Extraction

To isolate the phytochemicals from the bark of *Terminalia arjuna*, successive solvent extraction method was used [93]. This method employs treatment of various organic solvents to isolate phytochemicals of the same polarity as that of the organic solvent in which they are dissolved. Process of extraction was started

with 300 g of dried powder of the bark of the plant soaked in 6 liters of ethanol of strength 99.9% for 24 h. The extract was filtered through muslin cloth and was subjected to rotary evaporator at 65°C for 24 h to get a viscous liquid. This liquid was put in petri-plates at 40°C overnight. The dried extract so obtained was extracted with 150 ml of hexane, dichloromethane, ethyl acetate and n-butanol for 24 h in separating funnel successively. All the extracts were then dried in rotary evaporator at 40°C separately. The presence of total phenolic compounds of arjuna were expected in more polar solvents like ethyl acetate and butanol. Hexane and dichloromethane extracts were dissolved in 5 ml DMSO whereas ethyl acetate and butanol extracts were dissolved in 5 ml of double distilled water separately. Inhibitory potential of each fraction thus obtained was then checked on CaP assay and COM crystal growth *in vitro*.

Phytochemical screening

Various qualitative tests were then performed on all the dried fractions obtained after successive solvent extraction.

Test for Tannins

Presence of the tannins was confirmed by performing ferric chloride test. For this 0.5 gm of all extracts was boiled with 20 ml of double distilled water and filtered. A few drops 1% ferric chloride were added and observed for brownish green coloration.

Test for Saponins

The presence of saponins was determined by using 1 gm of all extracts boiled in 10 ml of double distilled water. Formation of froth on vigorous shaking and mixing of this froth with 3 drops of olive oil showing formation of an emulsion confirmed the presence of saponins.

Test for Flavanoids

To test the presence of flavanoids, 5 ml of diluted ammonia was added to all fractions mixed in double distilled water (10%) followed by addition of few drops of concentrated H_2SO_4. A yellow color formation indicated presence of flavanoids. This yellow color disappeared on standing.

Test for Terpenoids

To test the presence of terpenoids, Salkowski test was performed [94]. For this, 5 ml of each extract was mixed in 2 ml of chloroform and 3 ml of concentrated H_2SO_4 was carefully added to form a layer. Formation of reddish brown coloration of the interface confirmed the presence of terpenoids.

Qualitative test for the confirmation of saponins in butanol fraction by TLC

TLC (Thin Layer Chromatography) was performed by using 20×20 cm silica gel plate (0.25 mm silica gel). To this plate 15 µl of saponin rich butanol fraction obtained after successive solvent extraction of *Terminalia arjuna* bark was loaded. The solvent system used was butanol:water:acetic acid, $12 : 2 : 1$ (lower phase). Plates were sprayed with p-anisaldehyde:acetic acid:sulfuric acid $(1 : 2 : 100)$ and heated for 10 minutes at 110°C to visualize saponins appeared in the form of blue bands [95].

3.7 *In vivo* evaluation of antilithiatic properties of *Tamarindus indica* and *Terminalia arjuna*

To evaluate the antilithiatic properties of *Tamarindus indica*, its crude aqueous extract was given to the animals. However, anlithiatic behavior of *Terminalia*

arjuna was checked and confirmed by giving saponin rich butanol fraction obtained after bioassay guided successive solvent extraction of *Terminalia arjuna* bark. Administration of *Tamarindus indica* crude aqueous extract and saponin rich butanol fraction of *Terminalia arjuna* also confirmed the safety and efficacy of the biomolecules present if given to the living beings. The effectiveness of these two plants was tested on hyperoxaluric rat model in which urolithiasis was induced by giving ethylene glycol and ammonium chloride in their drinking water. Activity of both the plants was evaluated by examining serum, urine and kidney tissue after treatment.

For *in vivo* studies male rats of Wistar strain were procured from central animal house Panjab University, Chandigarh. 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 ethical guidelines for care and use of laboratory animals and were approved by the local care of Experimental Animal Committee.

3.7.1 Division of animals and respective treatments

A total of 128 animals were taken. These animals were divided into 2 groups, Group *A* and Group *B*. Group *A* animals were treated with crude aqueous extract *Tamaridus indica* and Group *B* animals were given saponin rich butanol fraction of *Terminalia arjuna*. Each group had 64 animals (Figure 3.1 and Table 3.2).

Group *A* animals – Evaluation of crude aqueous extract of *Tamarindus indica* on hyperoxaluric rats

Group *A* was further divided into 2 groups. Out of two groups one group was given 9 days treatment another group was given 15 days treatment. Each group

was again divided into 4 sub groups and each sub group had 8 animals in it.

- **Group *A1a***: Group *A1a* had control animals which were not given any treatment. They were provided with standard animal feed and water for 9 days.
- **Group *A1b***: The animals in *A1b* group were hyperoxaluric rats which were given 0.4% ethylene glycol with 1.0% ammonium chloride in their drinking water for 9 days.
- **Group *A1c***: The animals in this group were given dose - 1 i.e. 5% crude aqueous extract of *Tamarindus indica* along with 0.4% ethylene glycol and 1.0% ammonium chloride in their drinking water for 9 days.
- **Group *A1d***: The animals in this group were given dose - 2 i.e. 10% crude aqueous extract of *Tamarindus indica* along with 0.4% ethylene glycol and 1.0% ammonium chloride in their drinking water for 9 days.

Similar treatment was given to *A2a*, *A2b*, *A2c* and *A2d* animals but with a different time period. To these animals treatment period was of 15 days.

Group *B* animals – Evaluation of saponin rich butanol fraction of *Terminalia arjuna* obtained after bioassay guided successive solvent on hyperoxaluric rats

Group *B* was also further divided into 2 groups. Out of two groups one group was given 9 days treatment another group was given 15 days treatment. Each group was again divided into 4 sub groups and each sub group had 8 animals in it.

- **Group *B1a***: Group *B1a* had control animals which were not given any treatment. They were provided with standard animal feed and water for 9 days.

Name of the plant	Dose - 1	Dose - 2
Tamarindus indica	5% aqueous extract	10% aqueous extract
Terminalia arjuna	25 mg/kg body weight saponin rich n-butanol fraction	50 mg/kg body weight saponin rich n-butanol fraction

Table 3.2: Respective plant doses given to animals.

- **Group *B1b***: The animals in *B1b* group were hyperoxaluric rats which were given 0.4% ethylene glycol with 1.0% ammonium chloride in their drinking water for 9 days.
- **Group *B1c***: The animals in this group were given dose - 1 i.e. saponin-rich butanol fraction of *Terminalia arjuna* 25 mg/kg body weight intraperitoneally along with 0.4% ethylene glycol and 1.0% ammonium chloride in their drinking water for 9 days.
- **Group *B1d***: The animals in this group were given dose - 2 i.e. saponin rich butanol fraction of *Terminalia arjuna* 50 mg/kg body weight intraperitoneally along with 0.4% ethylene glycol and 1.0% ammonium chloride in their drinking water for 9 days.

Similar treatment was given to *B2a*, *B2b*, *B2c* and *B2d* animals but with a different time period. To these animals treatment period was of 15 days.

3.7.2 *In vivo* evaluation of effects of both the plants on hyperoxaluric rat model

3.7.2.1 Urine analysis

One day before sacrificing the animals i.e. on 8^{th} day and 14^{th} day of 9 days treatment and 15 days treatment respectively, animals were put in metabolic cages for 24 hrs to collect their urine samples. Urine was collected in a tube

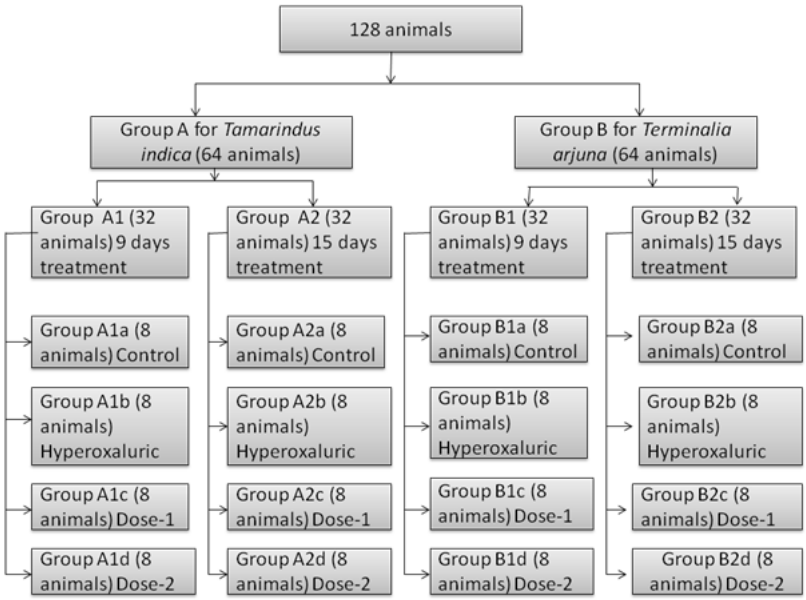

Figure 3.1: Flowchart representation of division of animals, their treatments and treatment time period.

attached to the funnel of the metabolic cages having 20 µl of 20% sodium azide as antibacterial and preservative. After collecting the urine an aliquot of urine was acidified by the addition of 3 N HCl for the determination of urinary creatinine. Remaining urine was stored at -20°C for determination of enzymes like alkaline phosphatase (AP) and lactate dehydrogenase (LDH).

Evaluation of creatinine content

It was estimated by the method of Bonsnes and Taussky (Bonsnes RW and Taussky HH, 1945).

Principle

Creatinine in alkaline solution reacts with picric acid to form an orange red compound. Rate of development of color is proportional to the concentration

of creatinine in the sample and absorbance of color was measured at 530 nm.

Reagents

- Picric acid: Saturated solution of picric acid was prepared by dissolving excess of picric acid in glass distilled water.
- Sodium hydroxide: 10 gm of $NaOH$ was dissolved in 250 ml of distilled water to prepare 1 M $NaOH$.
- Creatinine standard: Prepared by dissolving 10 mg of creatinine in 10 ml of distilled water.

Procedure

To create alkaline environment, 0.1 ml of test sample (acidified urine) was added into equal amount (0.1 ml) of 1 M $NaOH$. The urine sample and $NaOH$ was homogeneously mixed using vortex machine. To this alkaline urine sample 0.2 ml of picric acid solution was added. The solution was again mixed by vortex. Solution so formed was kept at room temperature for 10 minutes to allow the reaction between creatinine and picric acid. After 10 minutes of incubation at room temperature, the solution was diluted with 10 ml of distilled water and finally the absorbance was taken at 530 nm.

In addition, for blank and standard, similar procedure was followed and they were run parallel with the test sample. In blank, 0.1 ml of distilled water was added instead of urine sample. In standard, 0.1 ml of creatinine standard was added instead of urine sample.

Calculation

Concentration of creatinine was calculated (mg/dl) using following formula:

$$\text{Creatinine (mg/dl)} = \frac{\text{Absorbance of test}}{\text{Absorbance of standard}} \times \text{Concentration of standard}$$

Evaluation of Lactate dehydrogenase enzyme activity

Enzymatic activity of Lactate dehydrogenase was estimated by the method of Green [96].

Principle

Lactate dehydrogenase is cytoplasmic in its cellular location and in any one tissue is composed of one or two of five possible isoenzymes. LDH catalyses the readily reversible reaction involving the oxidation of lactate to pyruvate with NAD as cofactor.

$$\text{L-lactate} + \text{NAD} \leftrightarrows \text{pyruvate} + \text{NADH} + \text{H}^+$$

Enzyme activity is measured by estimating the concentration of $NADH$ present in the reaction mixture. $NADH$ absorbs light at 340 nm whereas NAD^+ does not. The equilibrium for the reaction lies very strongly in the direction of pyruvate and hence the reaction is followed by measuring the rate of decrease in the absorbance of $NADH$ at 340 nm.

Reagents

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

Procedure

3ml of substrate was taken in a cuvette. To this, 50 µl of $NADH$ and 200 µl urine test sample were added. After addition of all reagents, they were mixed rapidly and a decrease in absorbance was measured at 340 nm for 3 min.

Calculation

Activity was measured using following formula:

$$\text{Activity (units/mg protein)} = \frac{\text{Absorbance/min}}{6.22 \times \text{mg protein/ml of sample}}$$

Evaluation of alkaline phosphatase activity

Activity of alkaline phosphatase was measured by the method of Bergmeyer [97].

Principle

p-nitrophenyl phosphate was used as substrate. Alkaline phosphatase acts on this by hydrolyzing to yield p-nitrophenol. The yellow color was measured at 420 nm.

Reagents

- Buffered substrate: 375 mg glycine, 10 gm $MgCl_2$, 165 mg p-nitrophenyl phosphate were dissolved in 100 ml distilled water (pH 10.5)
- p-nitrophenol standard: 13.9 mg p-nitrophenol dissolved in 100 ml ditilled water.
- 0.1N $NaOH$: 0.4 gm of $NaOH$ dissolved in 100 ml of distilled water.
- Glycine buffer: 375 mg glycine dissolved in 42 ml of 0.1 N $NaOH$.

Procedure

Added 1 ml of buffered substrate to 50 µl of test sample. Volume was made to 0.5 ml by adding 450 µl of glycine buffer. This solution was incubated at room temperature for 15 min. After incubation, reaction was terminated by adding 3.5 ml of 0.1 N $NaOH$ and OD was measured at 420 nm. Blank and standard were also run parallel to test sample. In case of blank, distilled water and in case of standard p-nitrophenol standard was added in place urine sample.

Calculation

Activity was measured using following formula:

$$\text{Activity (units/mg protein)} = \frac{\text{OD of test}}{\text{OD of standard}} \times \text{OD of standard}$$

3.7.2.2 Serum analysis

Before sacrificing the rats on 9^{th} and 15^{th} day of 9 and 15 day treatment period respectively, the blood of rats was taken from orbital sinus into a centrifuge tube without anticoagulant and allowed to clot at room temperature for 15 minutes. The clotted blood was then centrifuged at 3000 rpm for 15 minutes to pellet out blood cells. The supernatant was collected as serum for the estimation of creatinine and urea.

Evaluation of creatinine content

The serum creatinine content was estimated by the method Bonseves and Taussky [98]. The urine test sample was replaced by serum test sample.

Evaluation of urea content

Urea in the serum was estimated by diacetylmonoxime method as described by Marsh [99].

Principle

Urea reacts with diacetylmonoxime under strongacidic conditions to give yellow condensation product. The reaction is intensified by the presence of ferric ion and thiosemicarbazide and red complex is formed whose absorbance is measured at 520 nm.

Reagents

1. Working urea standard: 200 mg of urea was dissolved in water and final volume was made to 100 ml.

2. The acid reagent was made by mixing stock A (0.5 ml) with 1 liter of stock B

   - Acid reagent (stock A): 5 gm of ferric chloride ($FeCl_3 6H_2O$) was dissolved in about 20 ml of water. This 20 ml solution was then transferred to a 250 ml measuring flask in which 100 ml of phosphoric acid (85%) was added slowly with swirling and the final volume was made to 250 ml with water.
   - Acid reagent (stock B): 200 ml of concentrated H_2SO_4 was added to 800 ml of water in one liter conical flask slowly, with swirling and cooling.

3. The color reagent was made by mixing 6.7 ml of stock A with 6.7 ml of stock B and made to 100 ml with double distilled water. Both the solutions were made fresh every time.

- Color reagent (stock A): 2 gm of Diacetyl monoxime was dissolved in 100 ml distilled water and then filtered to remove any suspended insoluble particles.
- Color reagent (stock B): 5 gm of thiosemicarbazide was dissolved with 1 liter distilled water.

Procedure

First of all the urine sample was diluted 100 times by distilled water. To 0.1 ml of urine sample 9.9 ml of distilled water was added. The diluted urine sample was thoroughly mixed on vortex. To this diluted urine sample, 2 ml of color reagent (reagent 3) was added and mixed. Followed by color reagent addition, same amount of mixed acid reagent (reagent 2) was added to the solution to impart acidic environment. The mixture of three solutions was mixed thoroughly in a vortex and the kept at 100°C for 20 minutes. After boiling water bath incubation the absorbance of the solution was read at 520 nm. Before reading absorbance, the contents of the tests tubes were cooled down. In addition, for blank and standard similar procedure was adopted and they were run parallel with test sample. In blank test tube, instead of urine test sample, 0.1 ml of distilled water was added and in case of standard, 0.1 ml of urea standard (reagent 1) was used.

Calculation

Concentration of urea (mg/dl) was calculated using following formula:

$$\text{Urea (mg/dl)} = \frac{\text{Absorbance of test}}{\text{Absorbance of standard}} \times \text{Concentration of standard}$$

Creatinine clearance

Creatinine clearance rate (CrCl) is the volume of blood plasma that is cleared of creatinine per unit time and is a useful measure for approximating the Glomerular filtration rate (GFR). Both GFR and CrCl may be accurately calculated by comparative measurements of substances in the blood and urine. Creatinine clearance was calculated according to standard clearance formula:

$$C = (U/S) \times V$$

where, U is the urinary concentration of creatinine, S is the concentration of creatinine in the serum, V is the volume of urine in ml/min.

3.7.2.3 Sacrificing animals

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

3.7.2.4 Statistical analysis

Statistical analysis was performed by unpaired, 2-tailed Student 't' test. It estimates whether the difference between the mean values of two groups are statistically significant or not.

3.7.2.5 Histological analysis

1. Fixation: Small pieces of kidney tissues, after removal of extraneous material were fixed in buffered saline formaldehyde. These were then dehydrated in various grades of ethanol, cleared in benzene and embedded in

paraffin wax (M. P. 60–62°C). The paraffin sections of appropriate thickness (8 µ) were cut.

2. Histological Staining [Delafield's Haemotoxylin/Eiosin (HE)]: First of all, the sections were de-paraffinised in the xylene and downgraded through different grades alcohols to water. These were then stained in D. Haematoxylin for 15 to 17 min. and then kept under running tap 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 the cytoplasm stained pink.

3. The stained slides were viewed under light microscope.

Chapter 4

Results

4.1 Effect of *Tamarindus indica* on *in vitro* mineralization reactions of calcium phosphate

First, the studies were conducted by using aqueous extract of *Tamarindus indica* on initial mineral phase formation, its subsequent growth and demineralization of preformed initial mineral phase formation. Effectiveness of the plant extract was evaluated by using different volumes of the aqueous extract of *Tamarindus indica* on 5 ml assay system. In case of initial mineral phase formation, maximum inhibition of 82% for calcium ion was observed with 1.0 ml aqueous extract of *Tamarindus indica* followed by 1.5, 0.8, 0.4 and 0.2 ml. For phosphate ion, maximum inhibition of 79% was shown by 0.4 ml of extract followed by 1.5, 1.0, 0.8 and 0.2 ml. Aqueous extract of *Tamarindus indica* resulted in maximum calcium ion inhibition of 53% and phosphate ions inhibition of 41% with 0.2 ml of extract when growth of preformed mineral phase was studied. Finally, demineralization was conducted and maximum amount of calcium and phosphate ions released were seen with 1.5 ml of aqueous extract of *Tamarindus indica* for

Extract volume used (ml)	Initial mineral phase formation		Growth		Demineralization	
	% inhibition of ions		% inhibition of ions		% release of ions	
	Calcium	Phosphate	Calcium	Phosphate	Calcium	Phosphate
0.05	---	---	15 ± 1.5	26 ± 1.8	---	---
0.1	---	---	16 ± 1.3	29 ± 1.4	---	---
0.15	---	---	43 ± 2.1	30 ± 2.1	---	---
0.2	12 ± 1.8	48 ± 1.8	53 ± 1.7	41 ± 2.0	104 ± 4.2	83 ± 3.1
0.4	67 ± 2.2	79 ± 2.5	--	--	256 ± 5.3	352 ± 5.1
0.8	68 ± 2.1	73 ± 1.5	--	--	385 ± 4.9	542 ± 4.3
1.0	82 ± 1.8	75 ± 1.5	--	--	387 ± 5.8	590 ± 5.8
1.5	78 ± 2.3	77 ± 1.9	--	--	412 ± 6.2	661 ± 6.8

Table 4.1: Effect of aqueous extract of *Tamarindus indica* on initial mineral phase formation, its subsequent growth and demineralization of preformed mineral phase. Results are expressed as mean ± SD of 5 different experiments.

both calcium and phosphate ions. Percentage release of ions was decreased with the decreased amount of aqueous extract. Hence, least release of ions was seen with 0.2 ml of aqueous extract of *Tamarindus indica* in case of demineralization. Table 4.1 depicts the effect of various volumes of the extract used on calcium and phosphate ion precipitation.

4.2 Effect of aqueous extract of *Tamarindus indica* on growth of calcium oxalate (CaOx) crystals

Aqueous extract of *Tamarindus indica* also showed significant inhibition for growth of $CaOx$ crystals. Studies were conducted with different volumes of the extract. Maximum inhibition was observed with 20 µl of aqueous extract followed by 80 µl, 60 µl, 10 µl and 40 µl as shown in Figure 4.1.

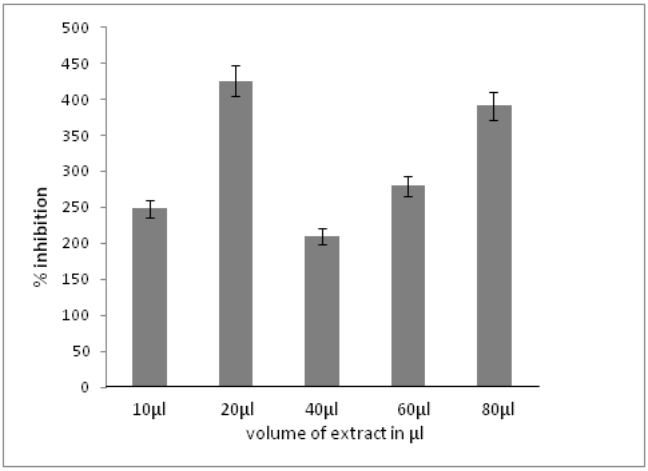

Figure 4.1: Effect of aqueous extract of *Tamarindus indica* on $CaOx$ crystal growth in terms of % inhibition of oxalate ions. Results are expressed as mean ± SD of 5 different experiments.

4.3 *In vivo* assessment of antilithiatic properties of *Tamarindus indica*

To elucidate the *in vivo* efficacy of *Tamarindus indica*, hyperoxaluric rat model was used. The evaluation was ascertained by administrating aqueous extract of *Tamarindus indica* to hyperoxaluric rats. Animals were divided into groups and sub groups according to the treatment given to them as described in the section 3.7.1. After the treatment period, the required samples were collected from the rats and various biochemical and histological parameters were estimated.

4.3.1 Urine examinations

The rats were examined daily for there general health and physical activity. The dose of EG and NH_4Cl and crude extract was well tolerable and rats progressed well during the course of treatment period.

4.3.1.1 Urinary alkaline phosphatase activity

The alkaline phosphatase activity in the urine was measured as a kidney injury marker enzyme. Figure 4.2 shows the activity of alkaline phosphatase (AP) enzyme in the urine sample of rats in all the groups. In case of 9 days treatment, the level of AP rose significantly in the hyperoxaluric *A1b* group ($p < 0.001$) by 228% when compared with control *A1a* group. The level of AP was reduced to 78.5% ($p < 0.001$) in *A1c* group when dose-1 i.e. 5% aqueous extract of *Tamarindus indica* along with EG and NH_4Cl was given and to rats and 7.1% in *A1d* group when dose-2 i.e. 10% aqueous extract of *Tamarindus indica* along with EG and NH_4Cl was given and to rats. No significant change was found in *A1d* group animals given dose-2 when compared with control *A1a*. This signifies that AP level of this group was almost completely normalized that of control group upon administration of dose-2 i.e. 10% aqueous extract of *Tamarindus indica* along with EG and NH_4Cl to rats.

In case of 15 days treatment, increased level of AP, 69% ($p < 0.05$) in hyperoxaluric *A2b* group was reduced to 40.6% when dose-1 i.e. 5% aqueous extract of *Tamarindus indica* along with EG and NH_4Cl was given to rats (*A2c*). The level of AP was further reduced to 25% when dose-2 i.e. 10% aqueous extract of *Tamarindus indica* along with EG and NH_4Cl was given to rats (*A2d*) (Figure 4.3).

4.3.1.2 Urinary Lactate Dehydrogenase activity

In case of 9 days treatment, the second renal injury marker enzyme, lactate dehydrogenase (LDH), showed a significant ($p < 0.001$) rose of 43.3% in hyperoxaluric *A1b* group when compared with control *A1a* group. This increased level was controlled in dose-1 and dose-2 group animals and was found to be 16.9% ($p < 0.01$) in *A1c* and 9.4% in *A1d* groups respectively when compared

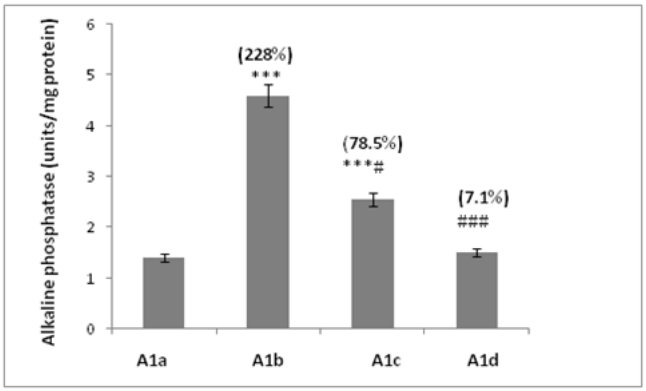

Figure 4.2: Activity of urinary alkaline phosphatase after 9 days of treatment. Values in brackets are percentage increase (+) or decrease (-) compared with control group (*A1a*); *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ indicates significant change in comparison with control group ; #$p < 0.05$, ## $p < 0.01$, ###$p < 0.001$ indicates significant change in comparison with hyperoxaluric group (*A1b*); *A1c* group is dose-1 animals given 5% aqueous extract and *A1d* group is dose-2 animals given 10% aqueous extract of *Tamarindus indica* along with EG and NH_4Cl.

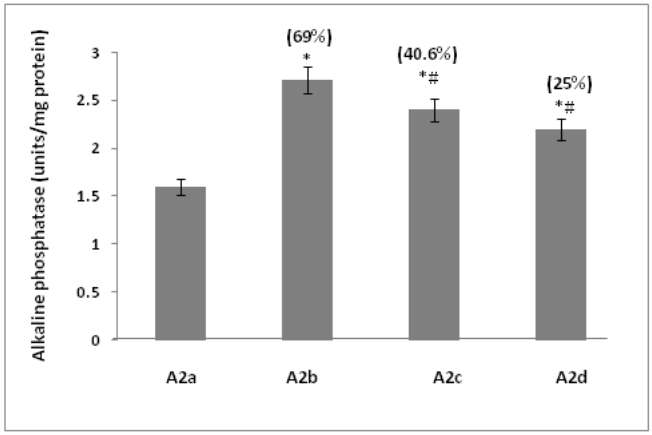

Figure 4.3: Activity of urinary alkaline phosphatase after 15 days of treatment. Values in brackets are percentage increase (+) or decrease (-) compared with control group (*A2a*); *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ indicates significant change in comparison with control group ; #$p < 0.05$, ## $p < 0.01$, ###$p < 0.001$ indicates significant change in comparison with hyperoxaluric group (*A2b*); *A2c* group is dose-1 animals given 5% aqueous extract and *A2d* group is dose-2 animals given 10% aqueous extract of *Tamarindus indica* along with EG and NH_4Cl.

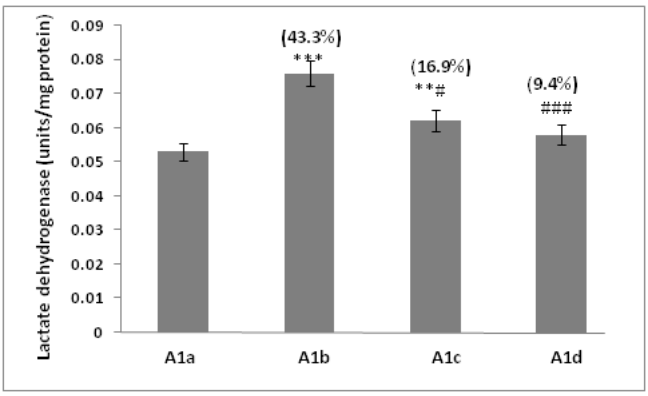

Figure 4.4: Activity of urinary lactate dehydrogenase after 9 days of treatment. Values in brackets are percentage increase (+) or decrease (-) compared with control group (*A1a*); $*p < 0.05$, $**p < 0.01$, $***p < 0.001$ indicates significant change in comparison with control group ; $\#p < 0.05$, $\#\#p < 0.01$, $\#\#\#p < 0.001$ indicates significant change in comparison with hyperoxaluric group (*A1b*); *A1c* group is dose-1 animals given 5% aqueous extract and *A1d* group is dose-2 animals given 10% aqueous extract of *Tamarindus indica* along with EG and NH_4Cl.

with control group. Again in dose-2 group *A1d* animals no significant change was found when compared with control group, concluding that LDH level was equivalent to control group animals (Figure 4.4).

In case of 15 days treatment also increased level of LDH, 10.2% ($p < 0.05$) in hyperoxaluric *A2b* group was reduced to 6.2% and 2.5% in dose-1 *A2c* and dose-2 *A2d* groups respectively when compared with control group (Figure 4.5).

4.3.2 Serum analysis

4.3.2.1 Serum urea

In case of treatment period of 9 days, serum urea level was increased significantly ($p < 0.001$) by 20% in hyperoxaluric (*A1b*) group when compared with control group (*A1a*). This increased level of serum urea was decreased to 8.5% and 2.8% when rats were treated with 5% and 10% crude extract of *Tamarindus*

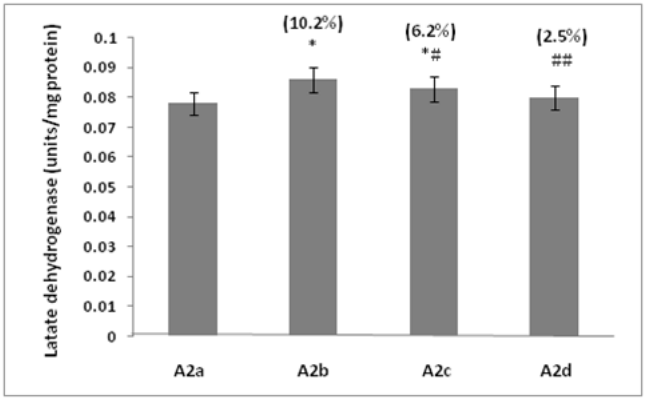

Figure 4.5: Activity of urinary lactate dehydrogenase after 15 days of treatment. Values in brackets are percentage increase (+) or decrease (-) compared with control group (*A2a*); $*p < 0.05$, $**p < 0.01$, $***p < 0.001$ indicates significant change in comparison with control group ; $\#p < 0.05$, $\#\#p < 0.01$, $\#\#\#p < 0.001$ indicates significant change in comparison with hyperoxaluric group (*A2b*); *A2c* group is dose-1 animals given 5% aqueous extract and *A2d* group is dose-2 animals given 10% aqueous extract of *Tamarindus indica* along with EG and NH_4Cl.

indica as shown in Figure 4.6.

Similarly, in 15 days treatment, serum urea level was checked. Serum urea level was found to be increased by 48.1% ($p < 0.001$) in hyperoxaluric (*A2b*) group when compared with control group (*A2a*). In dose-1 group this level was reduced significantly ($p < 0.001$) to 35.5%. In dose-2 group this level was further reduced to 3% again indicating normalization of 10% aqueous extract of *Tamarindus indica* along with EG and NH_4Cl to rats in *A2d* group (Figure 4.7).

4.3.2.2 Serum cretinine

Serum cretinine was further checked and an increased level of 16% ($p < 0.01$) was found in hyperoxaluric group when compared with control group. In dose-1 and dose-2 groups this level was reduced to 10% and 3.3% respectively (Figure 4.8).

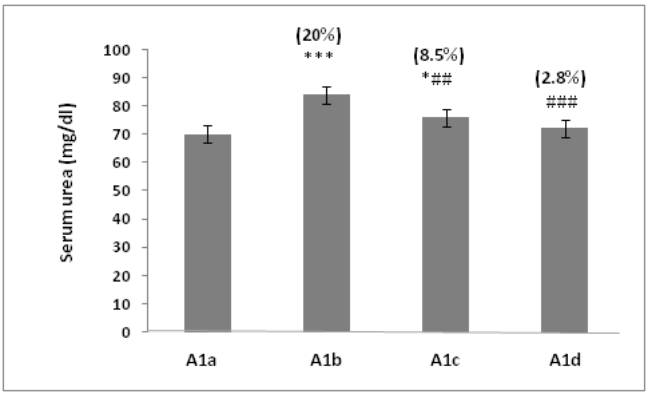

Figure 4.6: Content of serum urea after 9 days of treatment. Values in brackets are percentage increase (+) or decrease (-) compared with control group (*A1a*); $*p < 0.05$, $**p < 0.01$, $***p < 0.001$ indicates significant change in comparison with control group ; $\#p < 0.05$, $\#\#p < 0.01$, $\#\#\#p < 0.001$ indicates significant change in comparison with hyperoxaluric group (*A1b*); *A1c* group is dose-1 animals given 5% aqueous extract and *A1d* group is dose-2 animals given 10% aqueous extract of *Tamarindus indica* along with EG and NH_4Cl.

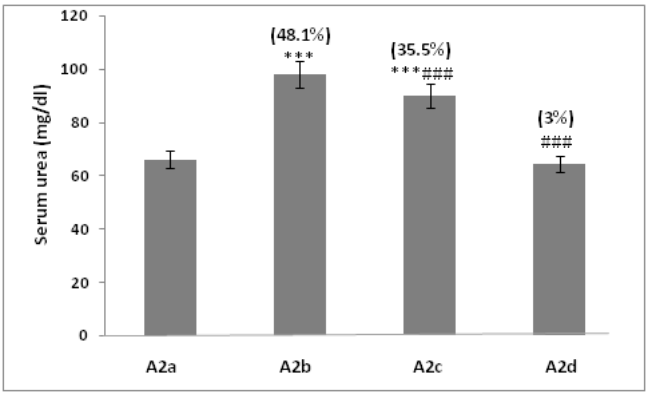

Figure 4.7: Content of serum urea after 15 days of treatment. Values in brackets are percentage increase (+) or decrease (-) compared with control group (*A2a*); $*p < 0.05$, $**p < 0.01$, $***p < 0.001$ indicates significant change in comparison with control group ; $\#p < 0.05$, $\#\#p < 0.01$, $\#\#\#p < 0.001$ indicates significant change in comparison with hyperoxaluric group (*A2b*); *A2c* group is dose-1 animals given 5% aqueous extract and *A2d* group is dose-2 animals given 10% aqueous extract of *Tamarindus indica* along with EG and NH_4Cl.

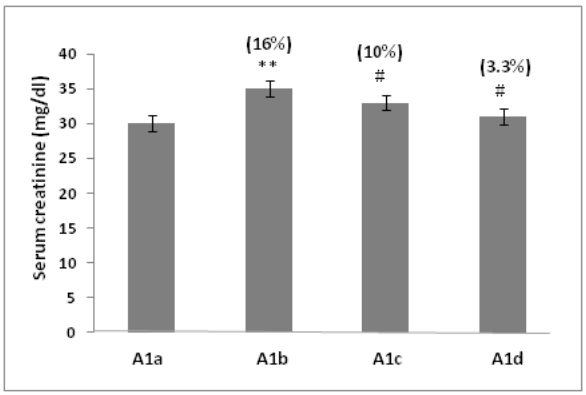

Figure 4.8: Content of serum creatinine after 9 days of treatment. Values in brackets are percentage increase (+) or decrease (-) compared with control group (*A1a*); $*p < 0.05$, $**p < 0.01$, $***p < 0.001$ indicates significant change in comparison with control group ; $\#p < 0.05$, $\#\#p < 0.01$, $\#\#\#p < 0.001$ indicates significant change in comparison with hyperoxaluric group (*A1b*); *A1c* group is dose-1 animals given 5% aqueous extract and *A1d* group is dose-2 animals given 10% aqueous extract of *Tamarindus indica* along with EG and NH_4Cl.

In case of 15 days treatment an increased level of 20% ($p < 0.05$) found in hyperoxaluric group (*A2b*) when compared with control group was normalized to 12% and 4% dose-1 and dose-2 were given to the animals in group *A2c* and *A2d* respectively w.r.t. control group (Figure 4.9).

4.3.2.3 Creatinine clearance

Creatinine clearance measured after 9 days treatment is shown in Figure 4.10. A significant decrease ($p < 0.001$) of 53.4% was observed in hyperoxaluric *A1b* group when compared with control *A1a* group animals. This level of clearance was increased in dose-1 and dose-2 group animals. In dose-1 group (*A1c*) animals, a significant change ($p < 0.05$) was found but in dose-2 group (*A1d*) animals clearance was similar as that of control group animals and hence no significant change was found in this group when compared with control group animals.

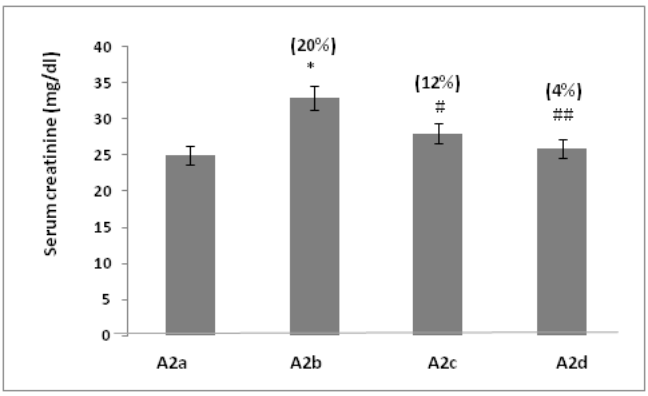

Figure 4.9: Content of serum creatinine after 15 days treatment. Values in brackets are percentage increase (+) or decrease (-) compared with control group (*A2a*); $*p < 0.05$, $**p < 0.01$, $***p < 0.001$ indicates significant change in comparison with control group ; $\#p < 0.05$, $\#\#p < 0.01$, $\#\#\#p < 0.001$ indicates significant change in comparison with hyperoxaluric group (*A2b*); *A2c* group is dose-1 animals given 5% aqueous extract and *A2d* group is dose-2 animals given 10% aqueous extract of *Tamarindus indica* along with EG and NH_4Cl.

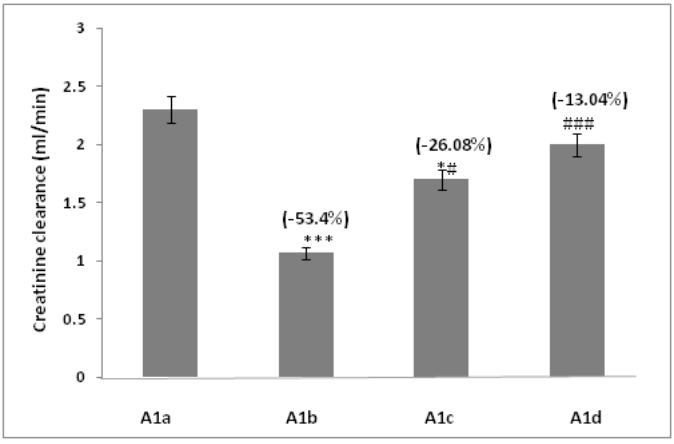

Figure 4.10: Level of creatinine clearance after 9 days of treatment. Values in brackets are percentage increase (+) or decrease (-) compared with control group (*A1a*); $*p < 0.05$, $**p < 0.01$, $***p < 0.001$ indicates significant change in comparison with control group ; $\#p < 0.05$, $\#\#p < 0.01$, $\#\#\#p < 0.001$ indicates significant change in comparison with hyperoxaluric group (*A1b*); *A1c* group is dose-1 animals given 5% aqueous extract and *A1d* group is dose-2 animals given 10% aqueous extract of *Tamarindus indica* along with EG and NH_4Cl.

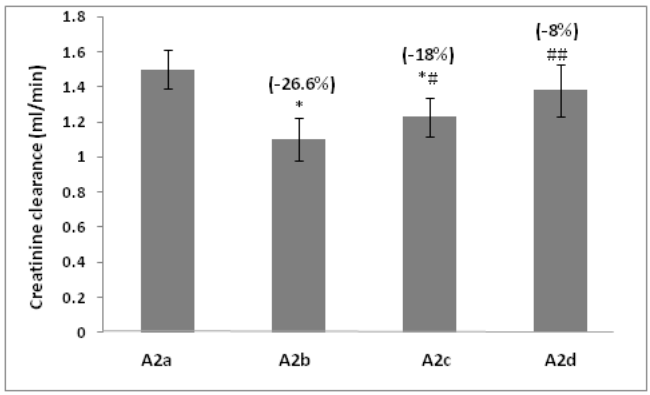

Figure 4.11: Level of creatinine clearance after 15 days of treatment. Values in brackets are percentage increase (+) or decrease (-) compared with control group (*A2a*); $*p < 0.05$, $**p < 0.01$, $***p < 0.001$ indicates significant change in comparison with control group ; $\#p < 0.05$, $\#\#p < 0.01$, $\#\#\#p < 0.001$ indicates significant change in comparison with hyperoxaluric group (*A2b*); *A2c* group is dose-1 animals given 5% aqueous extract and *A2d* group is dose-2 animals given 10% aqueous extract of *Tamarindus indica* along with EG and NH_4Cl.

Results of 15 days treatment are shown in Figure 4.11. On calculating creatinine clearance for 15 days treatment, a decrease of 26.6% was found in hyperoxaluric *A2b* group ($p < 0.001$) which was increased in dose-1 (*A2c*) and dose-2 (*A2d*) groups when compared with control group. A significant increase ($p < 0.05$) was found in dose-1 group when compared with control group but no change was found in dose-2 group again indicating normalization upon administration of 10% aqueous extract of *Tamarindus indica* extract along with EG and NH_4Cl to rats in *A2d* group.

4.3.3 Histological analysis of kidney tissue of rats treated with aqueous extract of *Tamarindus indica*

Transverse section of rat kidney tissue was taken and was stained with H & E dye and was viewed under light microscope. Control group animals in *A2a*

group were given no treatment so their kidneys showed normal morphology. The histology of control rats presented normal globular glomerulus within Bowman's capsule (Figure 4.12). Control group showed no sign of inflammation and hemolysis. Renal pelvic lining was also normal. On the other hand, the kidney histology of hyperoxaluric rats in *A2b* group showed a highly distorted morphology. The glomerulus had lost its globular characteristic and it appeared shrunken. The renal tubules had lost their intact structure as no distinct lumen was observed. In addition to these morphological alterations, the histology of rats in this group showed marked inflammation and deposits of blood protein casts in some tubules along with small mineral deposits in renal pelvis. In case of 9 days treatment, no significant change was observed after treatment neither in the morphology of the kidney nor in the mineral depositions in the kidney. However, in the case of rats given EG and NH_4Cl for 15 days, difference observed w.r.t. control group was remarkable as shown in Figure 4.13. In case of *A1b*, *A1c* and *A1d* group animals (in the case of rats given EG and NH_4Cl and aqueous extract of *Tamarindus indica* for 9 days) not much difference was seen in histology when they were compared with control group *A1a*.

The rats in the third group, *A2c* which were treated with 5% of crude aqueous extract of *Tamarindus indica* for 15 days along with EG and NH_4Cl showed a better morphology as compared to hyperoxaluric rats (Figure 4.14). In histology of these rats, the signs of inflammation were reduced and reduced hemolysis was observed. Though, the globular form of glomerulus was not restored completely. Among other signs of alterations, lymphomononuclear region in the cortex beneath the capsule was also visible. However, in case of forth group *A2d*, which was given 10% crude extract of *Tamarindus indica* along with EG and NH_4Cl their histology was comparable with that of control group animals (Figure 4.15). The glomerulus was of appropriate size and showed globular na-

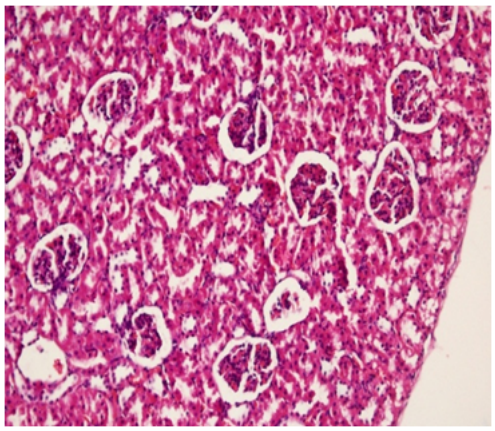

Figure 4.12: Control group after 15 days of treatment.

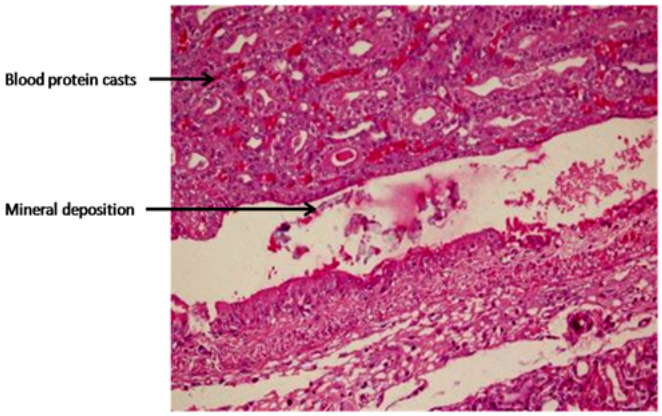

Figure 4.13: Hyperoxaluric group after 15 days of treatment.

ture to some extent. Here, in this case, mineralization also decreased to a great extent but still few deposits of mineral in the renal pelvis within a dilated tubule space were present. The renal histology of forth group, which was given 10% along with EG and NH_4Cl of crude extract of *Tamarindus indica*, was more near to control group than group three animals, which were given 5% of the crude aqueous extract of the *Tamarindus indica*, thus indicating that extract showed a dose-dependent response towards restoration of kidney.

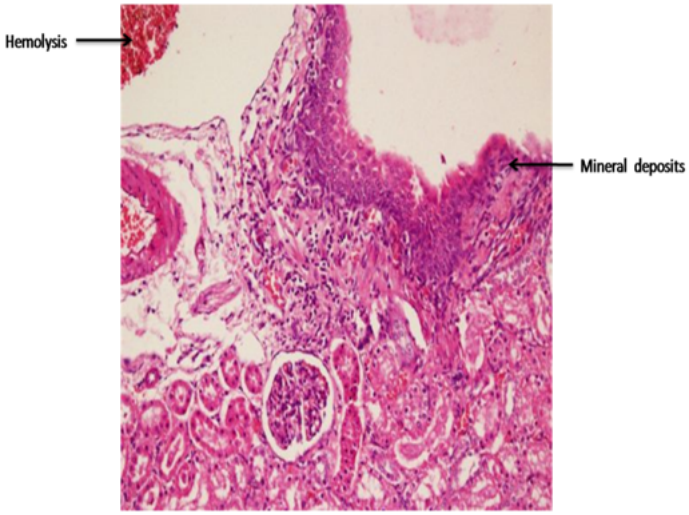

Figure 4.14: Animals treated with 5% of aqueous extract of *Tamarindus indica* after 15 days of treatment.

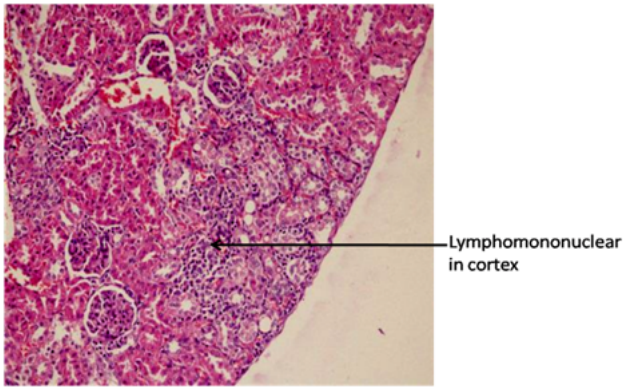

Figure 4.15: Animals treated with 10% of aqueous extract of *Tamarindus indica* after 15 days of treatment.

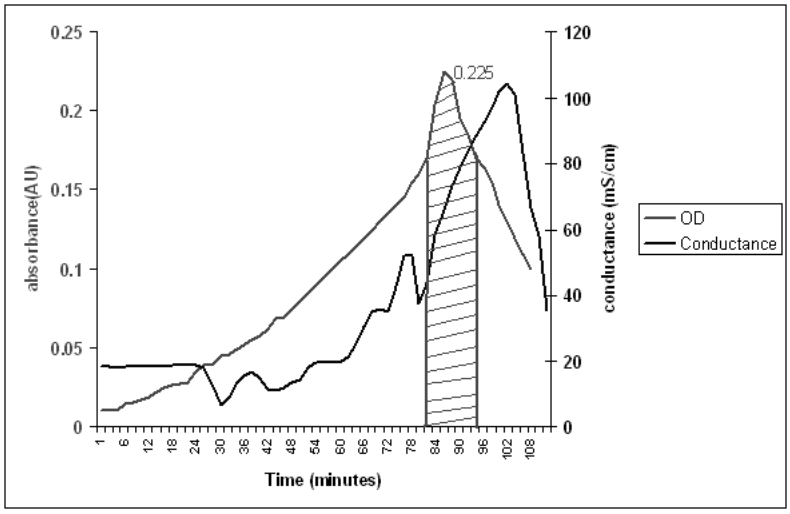

Figure 4.16: Elution profile of anion exchange chromatography of *Tamarindus indica* greater than 10 kDa fraction.

4.4 Protein purification of *Tamarindus indica*

Crude aqueous extract was then separated into two fractions (by Millipore ultrafilteration assembly having cut-off molecular weight filters of 10 kDa) having molecular weight less than 10 kDa and more than 10 kDa. Greater inhibition was shown by fraction having molecular weight more than 10 kDa, upto 360% in case of $CaOx$ crystal growth. Thus, further purification was continued with greater than 10 kDa fraction. Anion exchange chromatography was performed with greater than 10 kDa fraction. Activity of all the peaks obtained was checked against calcium phosphate (CaP) initial mineral phase formation, against nucleation of calcium oxalate ($CaOx$) crystals and against growth of $CaOx$ crystals. Out of all the protein peaks, most potent peak was observed at around 85 minutes having an absorbance of 0.225 (Figure 4.16).

The most potent peak (pooled fractions under shaded peak) showed a percentage inhibition of 19% for calcium and 11.5% for phosphate ions in case of CaP, 43% inhibition against nucleation of $CaOx$ crystals and 51% for oxalate

Purification Step	CaP mineralization		CaOx nucleation	CaOx crystal growth
	calcium	phosphate		
Anion exchange	19%	11.5%	43%	51%
Molecular sieve	28%	20%	48%	55%

Table 4.2: Percentage inhibition of calcium, phosphate and oxalate ions in CaP and $CaOx$ mineralization reactions after anion exchange chromatography and molecular sieve chromatography. Results are expressed as mean ± SD of 5 different experiments.

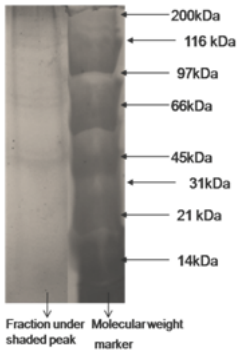

Figure 4.17: SDS-PAGE of shaded peak obtained after anion exchange chromatography.

ions in case of $CaOx$ crystal growth (Table 4.2).

Extent of purity of this shaded peak was further checked by running SDS-PAGE and bands were seen after Coomassie Brilliant Blue R-250 staining of the shaded peak (Figure 4.17).

Most potent purified fraction obtained after anion exchange chromatography was further purified by performing molecular sieve chromatography and three peaks were obtained (Figure 4.18).

Inhibitory activity was again checked of all the peaks obtained and it was found to be the maximum in case of peak-1 which was 28% for calcium and 20% for phosphate in case of CaP, 48% inhibition against nucleation of $CaOx$ crystals and 55% for oxalate ions in case of $CaOX$ crystal growth (Table 4.2).

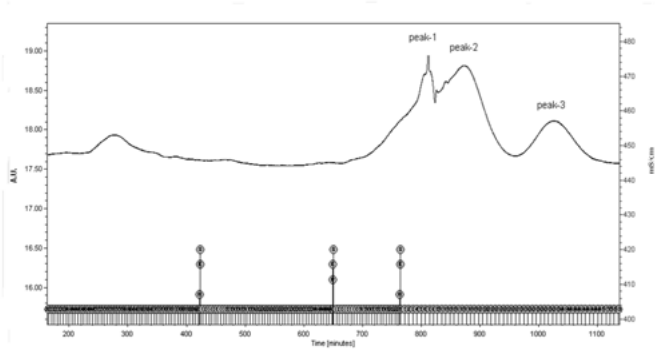

Figure 4.18: Elution profile of Molecular Sieve chromatography of shaded peak obtained after performing anion exchange chromatography.

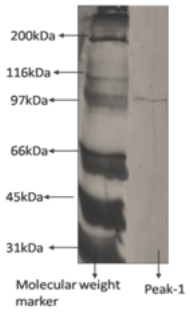

Figure 4.19: SDS-PAGE of peak-1 obtained after performing molecular sieve chromatography showing purified $\backsim$ 97 kDa protein.

Again SDS-PAGE of the most potent peak was run and a protein band having molecular weight $\backsim$ 97 kDa was visible. In Coomassie Brilliant Blue R-250 staining, no band was visible but after silver staining very light band was visible (Figure 4.19).

Volume of extract (ml)	Initial mineral phase formation		Growth		Demineralization	
	% inhibition of Ca^{2+} ions	% inhibition of HPO_4^{2-} ions	% inhibition of Ca^{2+} ions	% inhibition of HPO_4^{2-} ions	% release of Ca^{2+} ions	% release of HPO_4^{2-} ions
0.2	43 ± 3.3	68 ± 3.5	15 ± 1.1	50 ± 2.5	0	2 ± .15
0.4	14 ± 2.8	27 ± 2.8	36 ± 1.5	24 ± 1.6	30 ± 2.0	38 ± 2.3
0.8	15 ± 1.8	18 ± 1.9	37 ± 1.8	35 ± 2.3	80 ± 3.8	190 ± 2.8
1.0	18 ± 2.2	31 ± 1.8	41 ± 1.9	29 ± 2.2	135 ± 4.1	242 ± 6.8
1.5	48 ± 2.8	40 ± 2.0	48 ± 2.2	40 ± 2.5	197 ± 3.9	380 ± 7.1

Table 4.3: Effect of crude aqueous extract of *Terminalia arjuna* bark on initial mineral phase, its subsequent growth and demineralization of initial mineral phase formation. Results are expressed as mean ± SD of 5 different experiments.

4.5 Effect of aqueous extract of *Terminalia arjuna* on mineralization reactions of calcium phosphate *in vitro*

Assessment of effectiveness of *Terminalia arjuna* in management of urolithiasis was started with the treatment of aqueous extract of *Terminalia arjuna* bark on 5 ml system for calcium phosphate mineralization reactions. Table 4.3 depicts the effect of different volumes of aqueous extract *Terminalia arjuna* on initial mineral phase formation, its subsequent growth and demineralization of preformed initial mineral phase. Inhibition was seen in all the mineralization reactions. In case of initial mineral phase formation, maximum calcium and phosphate ion inhibition was observed with 1.5 ml and 0.2 ml respectively. Maximum inhibition of growth of calcium and phosphate ions was seen with 1.5 ml and 0.2 ml respectively. Maximum ion release of calcium and phosphate ions was seen with 1.5 ml.

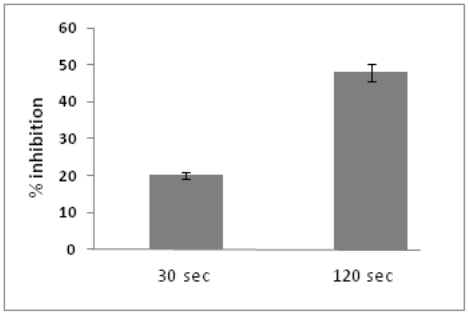

Figure 4.20: Effect of aqueous extract of *Terminalia arjuna* on $CaOx$ crystal growth. Results are expressed as mean ± SD of 5 different experiments.

4.6 Effect of aqueous extract of *Terminalia arjuna* on growth of calcium oxalate ($CaOx$) crystals

In case of $CaOx$ crystal growth, time scan of 2 minutes was run and a percentage inhibition of 20% was found at 30 sec. This inhibition was increased with the increase in time which was 48% at 120 sec when 15 µl of aqueous extract of *Terminalia arjuna* was used as shown in Figure 4.20.

4.7 Effect of fractions of *Terminalia arjuna* bark obtained after successive solvent extraction on calcium phosphate and calcium oxalate assay system

After studying the activity of crude aqueous extract of *Terminalia arjuna* on calcium phosphate and calcium oxalate assay system, it was fractionated by performing bioassay guided successive solvent extraction method. Various fractions

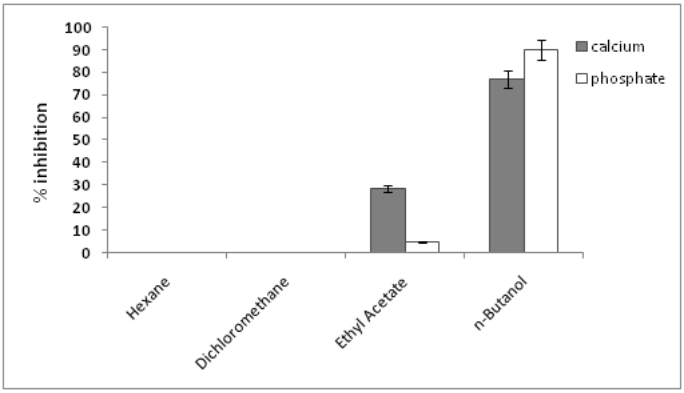

Figure 4.21: Effect of fractions obtained after successive solvent extraction of *Terminalia arjuna* on initial mineral phase formation of Calcium Phosphate (CaP) ions in terms of % inhibition. Results are expressed as mean ± SD of 5 different experiments.

were obtained and their inhibitory potential was checked first against calcium phosphate mineralization and then against calcium oxalate crystal growth. Out of all the fractions obtained viz. hexane, dichloromethane, ethyl acetate and n-butanol only ethyl acetate and n-butanol exhibited inhibitory activity whereas hexane and dichloromethane fractions did not show any inhibition for calcium and phosphate ion precipitation. In case of ethyl acetate, it showed a calcium inhibition of 28.26% and phosphate inhibition of 5% in initial mineral phase formation. However, n-butanol fraction showed enhanced percentage inhibition of 77% for calcium ions and 90% for phosphate ions when 0.1 ml of n-butanol fraction was used.

In case of $CaOx$ also, only n-butanol fraction showed inhibition of 29% and 69% at time intervals of 30 and 120 sec respectively as shown in Figure 4.22.

Phytochemical screening of all the fractions obtained was done after checking their inhibitory potency (Table 4.4). In ethyl acetate fraction presence of tannins and saponins was observed. Butanol fraction indicated presence of tannins and terpenoids in low amounts and saponins in very high amount. However,

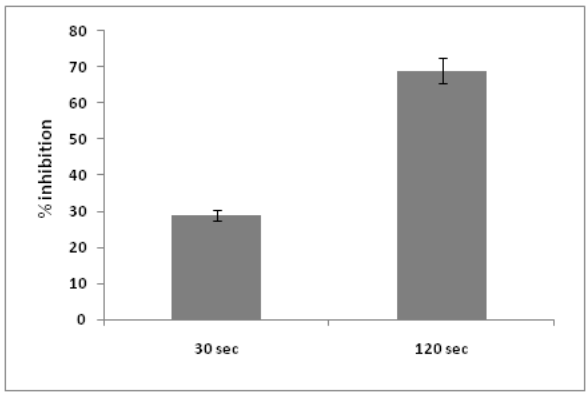

Figure 4.22: Effect of butanol fraction obtained after successive solvent extraction of *Terminalia arjuna* bark extract on *CaOx* crystal growth. Results are expressed as mean ± SD of 5 different experiments

	Tannins	Saponins	Terpenoids	Flavanoids
Hexane	(-)	(-)	(-)	(-)
Dichloromethane	Traces	(-)	(-)	(-)
Ethyl Acetate	(+)	(+)	(-)	(-)
n-Butanol	(+)	(+ +)	(+)	Traces

Table 4.4: Phytochemical screening of samples obtained after successive solvent extraction. (-): absent, (+): present in low amounts, (++): present in good amount.

hexane and dichloromethane fractions did not show marked presence of any of the phytochemical tested.

Further, thin layer chromatography of butanol fraction was done to confirm the presence of saponins and a blue colored band was seen as shown in Figure 4.23.

4.8 *In vivo* assessment of antilithiatic properties of *Terminalia arjuna*

To check the *in vivo* efficacy of *Terminalia arjuna* bark, again hyperoxaluric rat model was used. The evaluation was done by administrating saponin rich

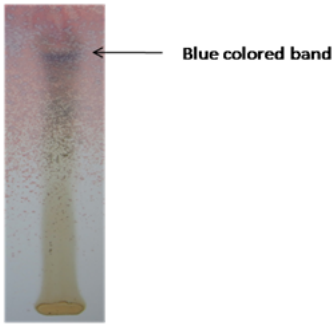

Figure 4.23: Detection of saponins in butanol fraction after running TLC.

butanol fraction of *Terminalia arjuna* bark obtained after successive solvent extraction method to hyperoxaluric rats. Animals were divided into groups and sub groups according to the treatment given to them as described in the section 3.7.1. After the treatment period, the required samples were again collected from the rats and various biochemical and histological parameters were estimated.

4.8.1 Urine analysis

Urine from all rats was collected in the metabolic cages one day before sacrificing them. Activity of two kidney injury marker enzymes, AP and LDH was evaluated in the urine sample.

4.8.1.1 Urinary alkaline phosphatase

In 9 days treatment, a significant increase ($p < 0.001$) of 140% was observed in the hyperoxaluric group (*A1b*) when compared with control group. This increased level was normalized to 62.96% in case of dose-1 (25 mg/kg body wt saponin rich butanol fraction of *Terminalia arjuna* bark along with EG and NH_4Cl) group animals (*B1c*). In case of dose-2 (50 mg/kg body wt saponin rich butanol fraction of *Terminalia arjuna* bark along with EG and NH_4Cl)

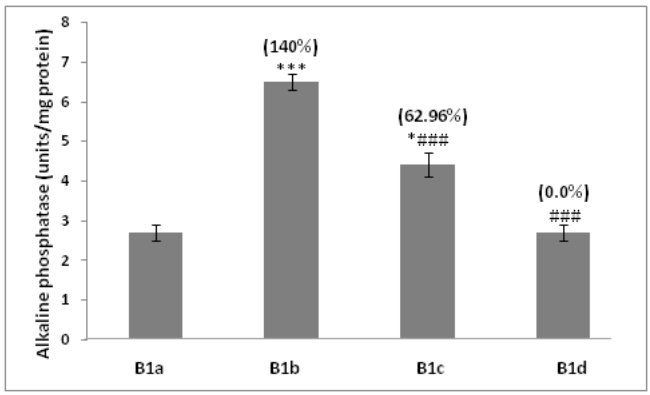

Figure 4.24: Activity of urinary alkaline phosphatase after 9 days of treatment. Values in brackets are percentage increase (+) or decrease (-) compared with control group (*B1a*); $*p < 0.05$, $**p < 0.01$, $***p < 0.001$ indicates significant change in comparison with control group ; $\#p < 0.05$, $\#\#p < 0.01$, $\#\#\#p < 0.001$ indicates significant change in comparison with hyperoxaluric group (*B1b*); *B1c* group is dose-1 animals given 25 mg/kg body wt saponin rich butanol fraction and *B1d* group is dose-2 animals given 50 mg/kg body wt saponin rich butanol fraction obtained after successive solvent extraction of *Terminalia arjuna* bark.

animals, 100% recovery was observed when compared with control group as shown in Figure 4.24.

In case of 15 days treatment, hyperoxaluric group (*B2b*) showed an increase of 140% in activity of enzyme activity with a significant change of $p < 0.001$ when compared with control group animals. After treating the animals with dose-1 (25 mg/kg body wt saponin rich butanol fraction of *Terminalia arjuna* bark) and dose-2 (50 mg/kg body wt saponin rich butanol fraction obtained after successive solvent extraction of *Terminalia arjuna* bark) this increased level was normalized to 90% and 30% respectively when compared with control group animals (Figure 4.25).

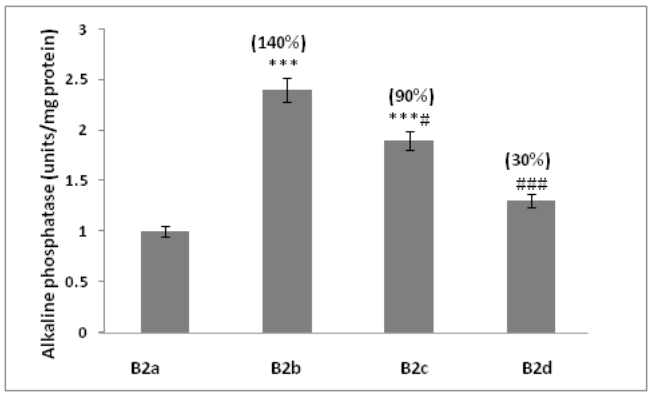

Figure 4.25: Activity of urinary alkaline phosphatase after 15 days of treatment. Values in brackets are percentage increase (+) or decrease (-) compared with control group (*B2a*); $*p < 0.05$, $**p < 0.01$, $***p < 0.001$ indicates significant change in comparison with control group ; $\#p < 0.05$, $\#\#p < 0.01$, $\#\#\#p < 0.001$ indicates significant change in comparison with hyperoxaluric group (*B2b*); *B2c* group is dose-1 animals given 25 mg/kg body wt saponin rich butanol fraction and *B2d* group is dose-2 animals given 50 mg/kg body wt saponin rich butanol fraction obtained after successive solvent extraction of *Terminalia arjuna* bark.

4.8.1.2 Urinary lactate dehydrogenase

Another kidney injury marker enzyme which was studied was lactate dehydrogenase. Increased level of lactate dehydrogenase was seen in hyperoxaluric group (*B1b*) in 9 days treatment. This level was significantly different ($p < 0.001$) from control group (*B1a*). The increased level was controlled to 27.6% and 7.6% by treating animals with 25 mg/kg body wt saponin rich butanol fraction for *B1c* group and 50 mg/kg body wt saponin rich butanol fraction along with EG and NH_4Cl for *B1d* animals respectively as shown in Figure 4.26.

Similarly, in case of 15 days treatment also increased level of enzyme activity in hyperoxaluric group (74.13%) was normalized to 62.06% and 24.13% when compared to control group animals (Figure 4.27).

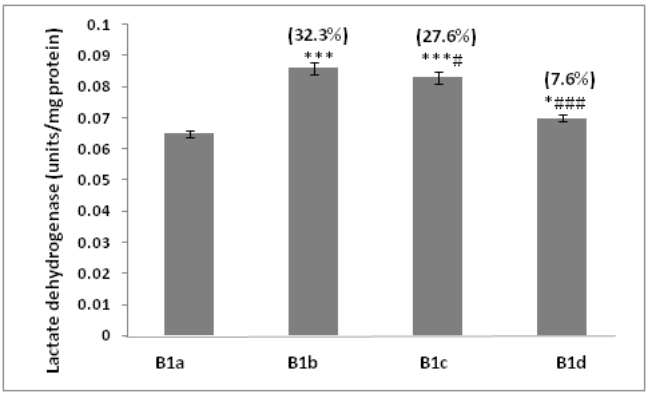

Figure 4.26: Activity of urinary lactate dehydrogenase after 9 days of treatment.Values in brackets are percentage increase (+) or decrease (-) compared with control group (*B1a*); $*p < 0.05$, $**p < 0.01$, $***p < 0.001$ indicates significant change in comparison with control group ; $\#p < 0.05$, $\#\#p < 0.01$, $\#\#\#p < 0.001$ indicates significant change in comparison with hyperoxaluric group (*B1b*); *B1c* group is dose-1 animals given 25 mg/kg body wt saponin rich butanol fraction and *B1d* group is dose-2 animals given 50 mg/kg body wt saponin rich butanol fraction obtained after successive solvent extraction of *Terminalia arjuna* bark.

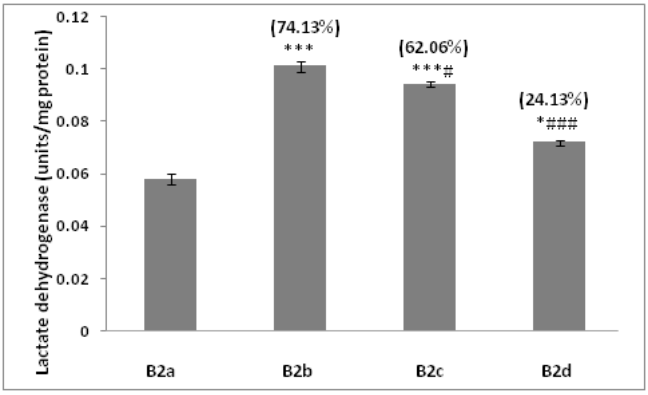

Figure 4.27: Activity of urinary lactate dehydrogenase after 15 days of treatment. Values in brackets are percentage increase (+) or decrease (-) compared with control group (*B2a*); $*p < 0.05$, $**p < 0.01$, $***p < 0.001$ indicates significant change in comparison with control group ; $\#p < 0.05$, $\#\#p < 0.01$, $\#\#\#p < 0.001$ indicates significant change in comparison with hyperoxaluric group (*B2b*); *B2c* group is dose-1 animals given 25 mg/kg body wt saponin rich butanol fraction and *B2d* group is dose-2 animals given 50 mg/kg body wt saponin rich butanol fraction obtained after successive solvent extraction of *Terminalia arjuna* bark.

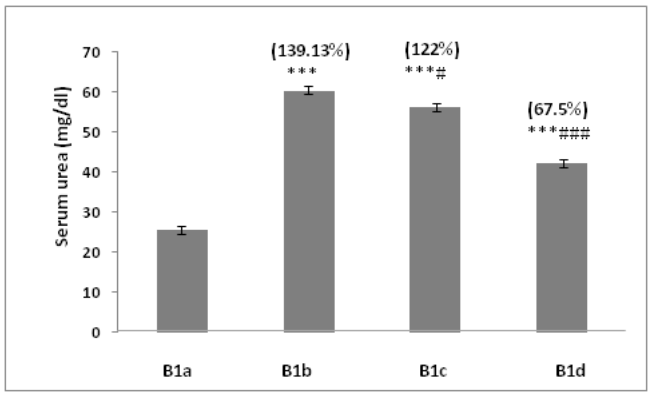

Figure 4.28: Content of serum urea after 9 days of treatment. Values in brackets are percentage increase (+) or decrease (-) compared with control group (*B1a*); $*p < 0.05$, $**p < 0.01$, $***p < 0.001$ indicates significant change in comparison with control group ; $\#p < 0.05$, $\#\#p < 0.01$, $\#\#\#p < 0.001$ indicates significant change in comparison with hyperoxaluric group (*B1b*); *B1c* group is dose-1 animals given 25 mg/kg body wt saponin rich butanol fraction and *B1d* group is dose-2 animals given 50 mg/kg body wt saponin rich butanol fraction obtained after successive solvent extraction of *Terminalia arjuna* bark.

4.8.2 Serum analysis

After collecting the serum estimation of serum urea and serum creatinine was done for 9 and 15 days treatment periods. At the end creatinine clearance was done and following results were obtained.

4.8.2.1 Serum urea

In case of 9 days treatment, hyperoxaluric group (*B1b*) showed a significant increase of 139.13%. Urea level in the serum was controlled by treating animals with plant dose. This increased level was found to be decreased to 122% in *B1c* group and 67.5% in *B1d* group animals as shown in Figure 4.28 when compared with control group.

Again in 15 days treatment, increased level of urea in serum in hyperoxaluric group (*B2b*) was normalized by treating animals with plant dose. After treat-

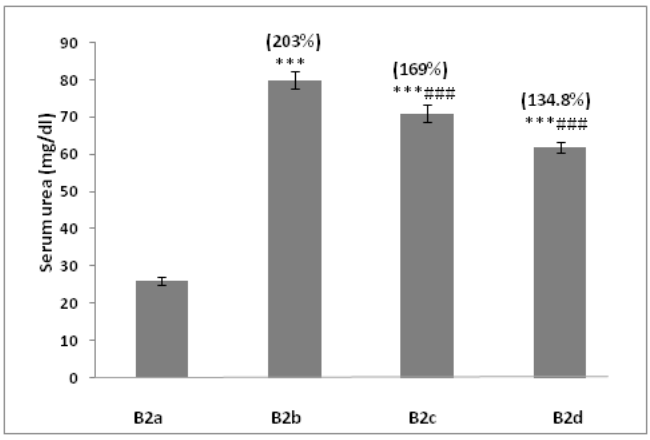

Figure 4.29: Content of serum urea after 15 days of treatment. Values in brackets are percentage increase (+) or decrease (-) compared with control group (*B2a*); $*p < 0.05$, $**p < 0.01$, $***p < 0.001$ indicates significant change in comparison with control group ; $\#p < 0.05$, $\#\#p < 0.01$, $\#\#\#p < 0.001$ indicates significant change in comparison with hyperoxaluric group (*B2b*); *B2c* group is dose-1 animals given 25 mg/kg body wt saponin rich butanol fraction and *B2d* group is dose-2 animals given 50 mg/kg body wt saponin rich butanol fraction obtained after successive solvent extraction of *Terminalia arjuna* bark.

ment, this level was found to be reduced to 169% in *B2c* group and 134.8% in *B2d* group animal (Figure 4.29).

4.8.2.2 Serum creatinine

After 9 days, hyperoxaluric group (*B1b*) showed increased level (66%, $p < 0.05$) of serum creatinine when compared with control group (*B1a*). However in case of *B1c* and *B1d* groups, increased level was found to be reduced to 42.8% and 14% respectively when compared with control group (Figure 4.30).

In case of 15 days treatment, increased levels (52%) of serum creatinine in hyperoxaluric group (*B1b*) were normalized to 20% and 4% in *B1c* and *B1d* groups respectively as shown in Figure 4.31.

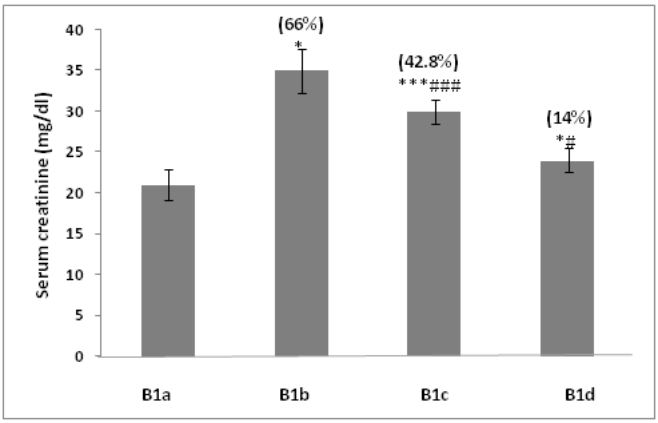

Figure 4.30: Content of serum creatinine after 9 days treatment. Values in brackets are percentage increase (+) or decrease (-) compared with control group (*B1a*); $*p < 0.05$, $**p < 0.01$, $***p < 0.001$ indicates significant change in comparison with control group ; $\#p < 0.05$, $\#\#p < 0.01$, $\#\#\#p < 0.001$ indicates significant change in comparison with hyperoxaluric group (*B1b*); *B1c* group is dose-1 animals given 25 mg/kg body wt saponin rich butanol fraction and *B1d* group is dose-2 animals given 50 mg/kg body wt saponin rich butanol fraction obtained after successive solvent extraction of *Terminalia arjuna* bark.

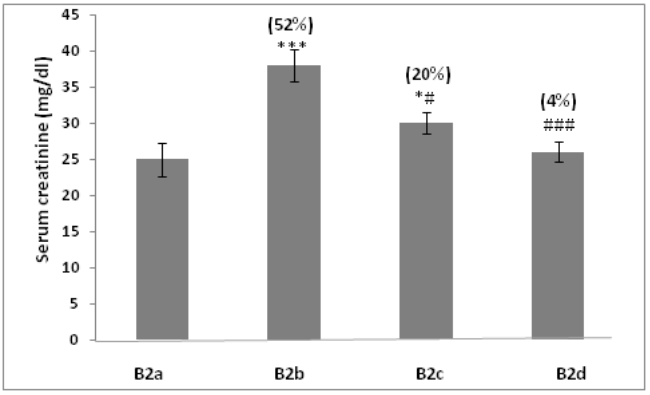

Figure 4.31: Content of serum creatinine after 15 days treatment. Values in brackets are percentage increase (+) or decrease (-) compared with control group (*B2a*); $*p < 0.05$, $**p < 0.01$, $***p < 0.001$ indicates significant change in comparison with control group ; $\#p < 0.05$, $\#\#p < 0.01$, $\#\#\#p < 0.001$ indicates significant change in comparison with hyperoxaluric group (*B2b*); *B2c* group is dose-1 animals given 25 mg/kg body wt saponin rich butanol fraction and *B2d* group is dose-2 animals given 50 mg/kg body wt saponin rich butanol fraction obtained after successive solvent extraction of *Terminalia arjuna* bark.

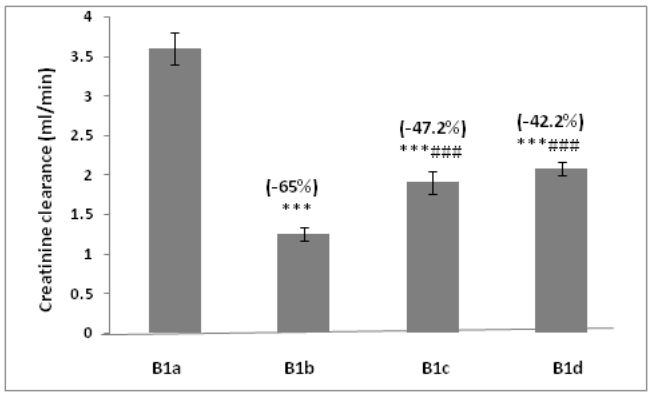

Figure 4.32: Level of creatinine clearance after 9 days of treatment. Values in brackets are percentage increase (+) or decrease (-) compared with control group (*B1a*); $*p < 0.05$, $**p < 0.01$, $***p < 0.001$ indicates significant change in comparison with control group ; $\#p < 0.05$, $\#\#p < 0.01$, $\#\#\#p < 0.001$ indicates significant change in comparison with hyperoxaluric group (*B1b*); *B1c* group is dose-1 animals given 25 mg/kg body wt saponin rich butanol fraction and *B1d* group is dose-2 animals given 50 mg/kg body wt saponin rich butanol fraction obtained after successive solvent extraction of *Terminalia arjuna* bark.

4.8.2.3 Creatinine clearance

Creatinine clearance measured in 9 days treatment is shown in Figure 4.32. A significant decrease ($p < 0.001$) of 65% was observed in hyperoxaluric *B1b* group when compared with control *B1a* group animals. This level of clearance was increased in dose-1 and dose-2 group animals. In both, dose-1 group (*B1c*) and dose-2 (*B1d*) group animals, a significant change ($p < 0.001$) was found when compared with control group animals.

Results of 15 days treatment are shown in Figure 4.33. On calculating creatinine clearance for 15 days treatment, a decrease of 27.7% was found in hyperoxaluric *B2b* group ($p < 0.05$) which was increased by 16.6% and 5.5% in dose-1 (*B2c*) and dose-2 (*B2d*) groups respectively when compared with control group. A significant increase ($p < 0.05$) was found in dose-1 group when compared with control group but no change was found in dose-2 group.

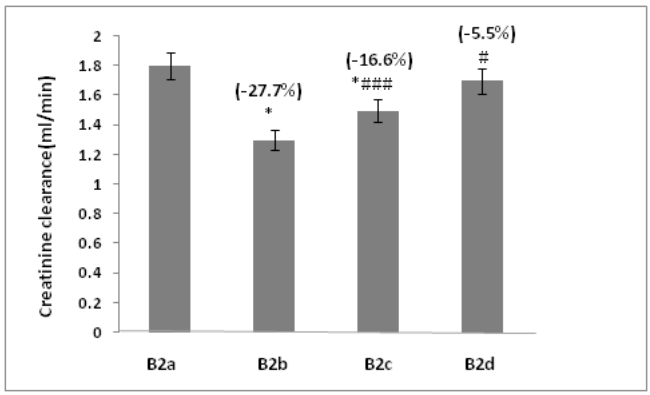

Figure 4.33: Level of creatinine clearance after 15 days treatment. Values in brackets are percentage increase (+) or decrease (-) compared with control group (*B2a*); $*p < 0.05$, $**p < 0.01$, $***p < 0.001$ indicates significant change in comparison with control group ; $\#p < 0.05$, $\#\#p < 0.01$, $\#\#\#p < 0.001$ indicates significant change in comparison with hyperoxaluric group (*B2b*); *B2c* group is dose-1 animals given 25 mg/kg body wt saponin rich butanol fraction and *B2d* group is dose-2 animals given 50 mg/kg body wt saponin rich butanol fraction obtained after successive solvent extraction of *Terminalia arjuna* bark.

4.9 Histological analysis of kidney tissue of rats treated with saponin rich butanol fraction obtained after successive solvent extraction of *Terminalia arjuna* bark

Transverse section of rat kidney tissue was taken from animals treated for 9 days and 15 days and was stained with H & E dye and was viewed under light microscope. Animals under 9 days treatment did not show any remarkable difference in histological analysis of the kidney tissue when viewed under light microscope. Control group animals under *B1a* and *B2a* groups showed normal physiology. The histology of control group showed normal globular glomerulus within Bowman's capsule. The histology of control group, showed no sign of inflammation and hemolysis (Figure 4.34). On the other hand, histology of hyperoxaluric rats

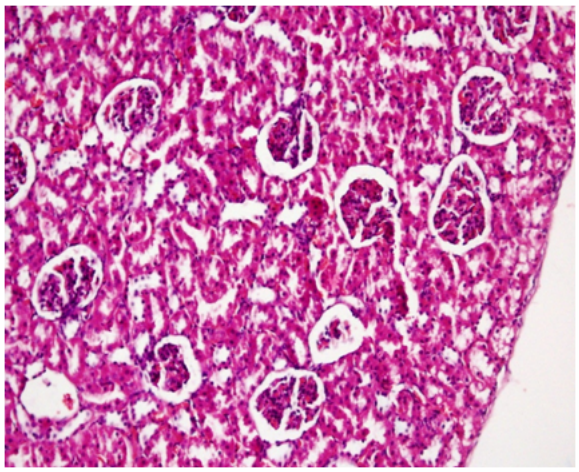

Figure 4.34: Control group after 15 days of treatment.

in group *B2b* showed a highly distorted morphology. Epithelial lining of Bowman's capsule was found to be broken at many instances. In addition to that, mineral deposition and hemolysis was also visible as shown in Figure 4.35.

The rats in *B2c* group which were treated with saponin rich butanol fraction at a dose of 25 mg/kg body weight portrayed a restored morphology as compared to control group (Figure 4.36). Reduced signs of inflammation and reduced mineral deposition were seen in the animals of this group. In *B2d* group animals which were given saponin rich butanol fraction at a dose of 50 mg/kg body weight, showed better morphology as compared to *B2b* and *B2c* group animals. Epithelial lining of Bowman's capsule was recovered with very less mineral deposits. Hemolysis and protein casts were also normalized and were quite comparable to control group animals. The decrease in tissue injury was also evidently observed with normal glomerulus (Figure 4.37)

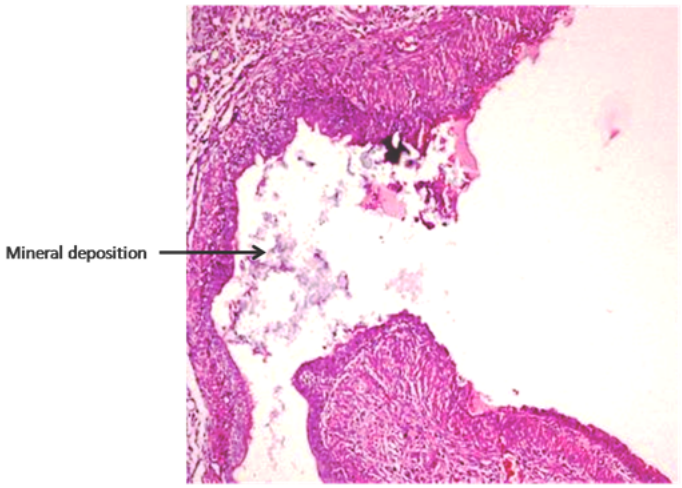

Figure 4.35: Hyperoxaluric group after 15 days of treatment.

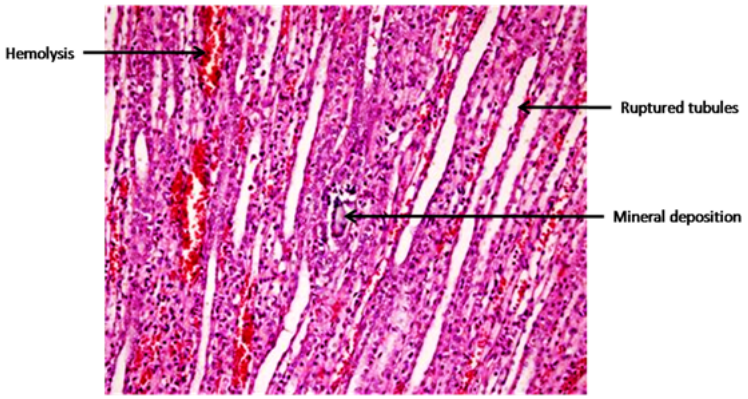

Figure 4.36: Animals treated with 25 mg/kg body weight of saponin rich butanol fraction obtained after successive solvent extraction of *Terminalia arjuna* bark after 15 days of treatment.

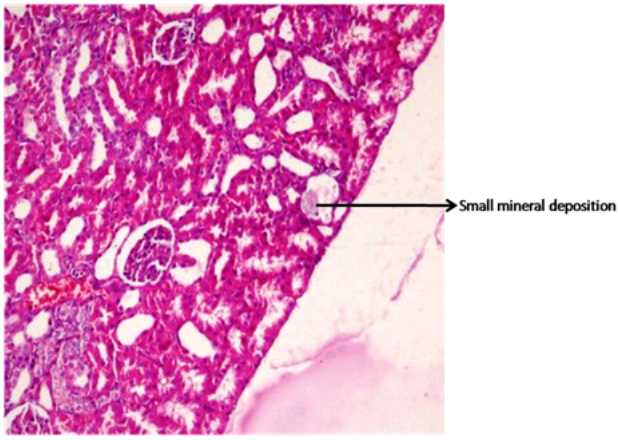

Figure 4.37: Animals treated with 50 mg/kg body weight of saponin rich butanol fraction obtained after successive solvent extraction of *Terminalia arjuna* bark after 15 days of treatment.

4.10 Protein purification of *Terminalia arjuna* bark

In order to purify the proteins present in the aqueous extract of *Terminalia arjuna* bark having the ability to influence CaP and $CaOx$ mineralization, ultrafilteration of the crude aqueous extract of the plant was performed by using Millipore ultrafilteration assembly having cut-off molecular weight filters of 10 kDa. Effect of both fractions, having molecular weight less than 10 kDa and greater than 10 kDa along with the crude extract, was checked on CaP crystal mineralization, $CaOx$ nucleation and $CaOx$ growth. Further purification was carried out by using fraction having molecular weight more than 10 kDa as it showed maximum inhibition. Purification was further sustained by running column chromatography techniques. First, anion exchange chromatography was performed and peaks were obtained. Out of all the peaks, the shaded peak showed maximum inhibition (Figure 4.38).

Anion exchange chromatography was followed by molecular sieve chromatography and following peaks were obtained. Out of all the peaks, peak - 3 showed

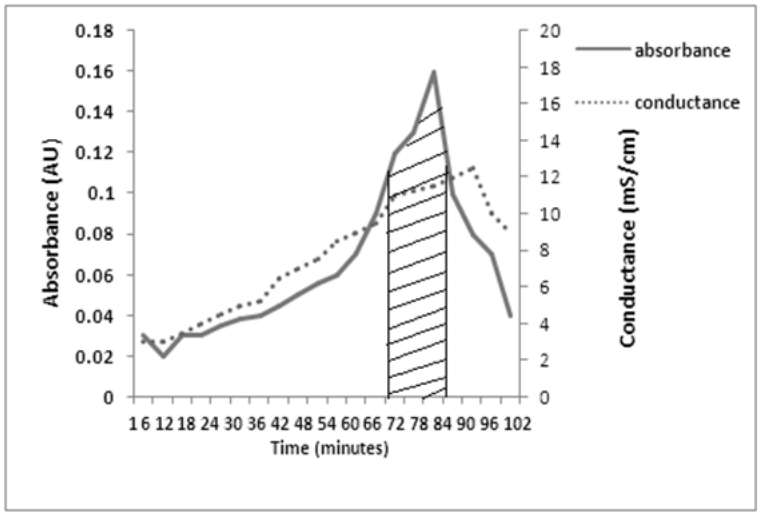

Figure 4.38: Elution profile of anion exchange chromatography of *Terminalia arjuna* greater than 10 kDa fraction.

maximum inhibition. Enhanced inhibition was seen after each step of purification (Figure 4.39). Enhanced inhibition was seen after each step of purification as shown in Table 4.5.

Extent of purity of protein fraction so obtained was validated by running 10% polyacrylamide gel and bands were visualized after performing silver staining. Thus, a protein band of molecular weight of $\backsim$ 14 kDa was purified (Figure

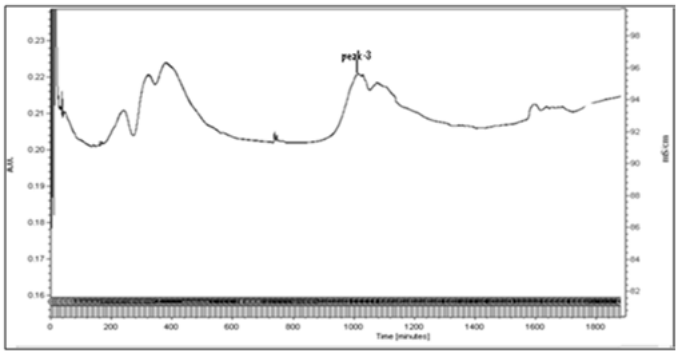

Figure 4.39: Elution profile of molecular sieve chromatography.

Purification step	CaP mineralization		CaOx nucleation	CaOx growth
	calcium	phosphate		
>10kDa fraction	70%	55%	75%	60%
Anion exchange	73%	65%	76%	69%
Molecular sieve	75%	72%	80%	78%

Table 4.5: Percentage inhibitions showed by all fractions obtained after each step of purification. Results are expressed as mean ± SD of 5 different experiments.

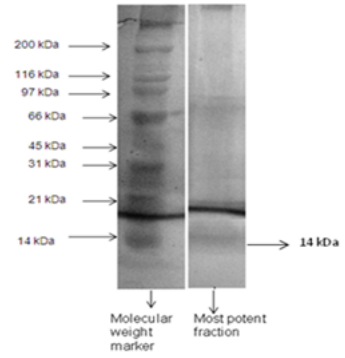

Figure 4.40: Silver stained 10% polyacrylamide gel showing protein band of molecular ∽ 14 kDa

4.40).

Chapter 5

Discussion

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

The majority of upper urinary stones are composed of calcium oxalate and calcium phosphate and usually occur in men, while most stones of magnesium and ammonium phosphate occur in the bladder, mostly in women [103]. Kidney stones often occur when urine becomes too concentrated [7]. This causes calcium, oxalate and phosphate or other chemicals in the urine to form crystals

on the inner surfaces of kidneys. This stage is called as initial mineral phase formation. Over the time, these crystals may combine to form a small, hard mass called as stones and stage is referred to as subsequent growth of crystals. Besides this, an uneven balance of acid in the urine and lack of substances in the urine that prevent the growth of crystals also affects the ability of stone forming substances to remain dissolved.

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

tilithiatic behavior of *Tamarindus indica* (fruit) and *Terminalia arjuna* (bark) has been studied both *in vitro* and *in vivo*. First, the homogenous systems of *in vitro* mineralization were employed under physiological conditions of temperature, pH and ionic strength of the media to see the effect of *Tamarindus indica* and *Terminalia arjuna* on CaP initial mineral phase formation, its subsequent growth and demineralization and on growth of $CaOx$ crystals. Both the plants were found to be inhibitory against urolithiasis.

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

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

Tamarindus indica exhibited inhibition against various stages of stone formation. It showed inhibition against initial mineral phase formation, its subsequent growth and and demineralization of CaP and against growth of $CaOx$ crystals *in vitro*. After confirming the antilithiatic nature *in vitro*, further validation was done *in vivo* on experimentally induced hyperoxaluric rat models. The rats induced with EG and NH_4Cl showed a marked elevation of urinary injury marker

enzymes (LDH and AP). This suggests damage to the brush border membrane of renal tubule, which appears to be associated with the retention and deposition of crystals in the kidney [115]. This excretion was normalized by putting animals on plant dose clearly showing antilithiatic properties of *Tamarindus indica* fruit. Further, creatinine clearance was observed, which is a useful measure for determining renal functioning. The impairment of renal functioning after treating animals with EG and NH_4Cl is an outcome of calcium oxalate crystals deposition in renal tissue. The effect of EG and NH_4Cl on renal functioning has been studied by many study groups [116]. This impairment was ameliorated when 5% and 10% of crude aqueous extract of the plant was given to the animals. Improvement in renal injury was well supported by histopathological studies. Decreased renal injury decreases sites for calcium oxalate deposition. Administration of crude plant extract to hyperoxaluric rats, prevents supersaturation of calcium oxalate and thus decreased their deposition in renal tubules.

Citrate and tartarate are known as good inhibitors of kidney stones [117]. In *Tamarindus indica*, both of them are present, which may contribute to its antilithiatic properties [118]. Studies have been done showing tartaric acid as inhibitor of CaP stones. Tartrates are expected to form metal ion complexes with calcium. Tartrates bind with the cations needed for crystal formation and subsequent growth, and also function as a crystal growth inhibitor of calcium oxalate by chemical adsorption on the crystallization sites at a growing interface [119]. An evaluation of tamarind and tartaric acid as inhibitor of calcium oxalate crystallization in urine is also reported [120]. Isolation and purification of the biomolecule(s) present in the aqueous extract of *Tamarindus indica* which was responsible for the antilithiatic properties was started with ultrafiltration. Crude aqueous extract was separated into two fractions, one having molecular weight less than 10 kDa and another having molecular weight greater than

10 kDa. Greater than 10 kDa fraction showed better inhibition and was further purified by applying anion exchange chromatography and molecular sieve chromatography. Protein fraction so obtained was loaded in SDS-PAGE and an anionic protein band having molecular weight of $\backsim$ 97 kDa was seen. Further, it has been seen that out of all the biomolecules present in *Tamarindus indica*, proteins make up nearly 8% [121]. Medicinal plants viz. *Trachyspermum ammi* [110] and *Dolichos biflorus (L.)* [111] are also shown to have antilithiatic proteins in them.

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

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

the atherosclerotic plaque could be attributed to the antioxidant property or to the calcium channel blocking property of *Terminalia arjuna* [125].

Apart from this, *Terminalia arjuna* is also well known for its high phenolic content. Thus, in the present study, bioactivity guided successive solvent extraction method was used to fractionate bioactive compounds from *Terminalia arjuna*. Here, bioactive compounds were separated on the basis of their polarity [93]. After performing froth test for the presence of saponins in butanol fraction, TLC showed the blue colored band confirming the presence of saponins. Saponin rich fractions of other plants like, *Herniaria hirsute* [126] has also been found to be a good inhibitor of calcium stone formation not only *in vitro* but *in vivo* too as found in present study. Saponin rich butanol fraction thus obtained after successive solvent extraction was administered to hyperoxaluric rats exposed to EG + NH_4Cl. Series of experiments done on urine and serum suggested preventive nature of saponin rich butanol fraction against urolithiasis. The results so obtained were very well supported by histopathological studies done with rat kidney tissue. Treated rats showed morphology like that of control group animals which were not given any treatment. In case of 9 days of treatment saponin rich butanol fraction showed 100% recovery when activity of alkaline phosphatase, a kidney injury marker enzyme, was measured. Main mode of action of inhibitors can be attributed to the structural fit between the biomolecule and the ionic structure of a particular crystal face. This decides the order of inhibition or reaction at a particular crystalline face. It may affect various crystalline faces differently depending upon the crystalline face exposed to the solution. As a result, it may change the morphology of the growing crystal. Small molecules with several negatively charged groups, such as citrate ions, do interact with the lateral faces of CaP and $CaOx$ crystals. They slow down crystallization and invite changes in the crystal morphology.

Apart from this, there are various stone inhibitory proteins [127, 128] which are present in urine, having similar physical and chemical properties. Most of these proteins have been isolated from $CaOx$ kidney stones matrix itself in their active form [129]. Likewise, many plants are also known to produce $CaOx$ as crystalline deposits [130] having an organic matrix constituting different proteins. These proteins are believed to play an important role in the control of crystal growth and modification of crystal form. Thus, protein purification of *Terminalia arjuna* was further done. Again ultrafiltration was done and further purification was continued with greater than 10 kDa fraction. Enhanced inhibition was seen after each step of purification. Extent of purity was checked by running 10% SDS-PAGE of most potent fraction obtained after molecular sieve chromatography and an anionic protein having molecular weight $\backsim$ 14 kDa was isolated. Since, the proteins present in both *Tamarindus indica* and *Terminalia arjuna* inhibited CaP as well as $CaOx$ crystal growth, a detailed study on the characterization of these proteins can impart a new direction to the treatment and cure of kidney stones.

Chapter 6

Summary and conclusions

Urinary tract and kidney stones ailments have affected human beings since antiquity. Ancient Vedic literature describes stones as Ashmari. Stones are solid concretions or calculi (crystal aggregations) formed in the kidneys from dissolved urinary minerals when urine becomes supersaturated. The occurrence of these stones has been increasing in rural and urban societies [131]. A large population of India suffers from urinary tract and kidney stones, formed due to the deposition of calcium, phosphate and oxalate. The chemicals start accumulating over the nucleus, which ultimately takes the shape of a stone.

Kidney stones modify the victim's behavior with great fear of intense pain and threat of kidney failure. In modern medicine no satisfactory effective therapy is still available to dissolve or to prevent stones. Acetozolamide are available against uric acid and cystine stone, although several drugs / therapies such as thiazides, cellulose phosphate, magnesium oxide and pyridoxine etc. have been tried. On the other hand, traditional system of Indian medicine "Ayurveda" recommends several medicinal plants for the treatment of Urolithiasis.

Urinary stones contain both crystalloid and colloid components. The crys-

talloid components are mainly calcium oxalate, calcium phosphate, calcium carbonate, magnesium-ammonium phosphate, uric acid and cysteine. Since ancient times, a variety of herbal preparations have been successfully used in renal lithiasis therapy [132, 133, 134]. During recent decades, studies of the antilithiatic effects of herbal extracts have been reported, but in the majority of these reports, the effects did not seem to be mediated by urinary biochemical changes [45, 135]. In the present study antilithiatic properties of two plants, *Tamarindus indica* and *Terminalia arjuna* have been assessed using *in vitro* and *in vivo* experimental models.

1. Effect of both the plants was first studied on calcium phosphate mineralization and on growth of calcium oxalate crystals *in vitro* by using different assay systems. It was found that both *Tamarindus indica* and *Terminalia arjuna* are effective not only in inhibiting initial mineral phase formation, growth and demineralization of the preformed mineral phase of CaP, but also in inhibiting $CaOx$ crystal growth *in vitro*. Crude aqueous extract of *Tamarindus indica* and *Terminalia arjuna* were then subjected to ultrafiltration and two fractions having molecular weight less than 10 kDa and greater than 10 kDa were obtained. Greater than 10 kDa fraction showed greater inhibition and thus protein purification was carried out with greater than 10 kDa fraction.

2. *In vivo* efficacy of *Tamarindus indica* was tested by using aqueous extract on experimentally induced hyperoxaluric rat models. Creatinine clearance, urinary injury marker enzymes and content of serum urea and creatinine levels were restored in animals treated with 5% and 10% aqueous extract of *Tamarindus indica* separately. Histopathological studies further showed marked decrease in crystallization when kidney tissue was seen under light microscope. Consumption of *Tamarindus indica* might be the reason of

low occurrence rate of kidney stones in the areas where its consumption is high.

3. Protein purification of *Tamarindus indica* done by anion exchange chromatography and molecular sieve chromatography carried out with greater than 10 kDa fraction revealed that it has a anionic protein (MW $\backsim$ 97 kDa) which is an effective inhibitor of calcium phosphate and calcium oxalate crystals.

4. *In vivo* efficacy of *Terminalia arjuna* was checked by treating hyperoxaluric rats with saponin rich n-butanol fraction obtained after bioactivity guided successive solvent extraction from the bark of *Terminalia arjuna*. Parameters studied on serum and urine revealed that saponin rich n-butanol fraction of *Terminalia arjuna*, reduced oxalate mediated manifestations in rat kidney. Decrease mineral deposits were observed in kidney tissue when seen under light microscope. In addition to that, less haemolysis and protein casts were seen in rats treated with saponin rich n-butanol fraction. Morphology was also restored in rats treated with saponin rich n-butanol fraction of *Terminalia arjuna*.

5. Protein purification of greater than 10 kDa fraction of crude aqueous extract of *Terminalia arjuna* was done and an anionic inhibitory protein (MW $\backsim$ 14 kDa) was isolated which is capable of inhibiting kidney stone formation.

Hence, both *Tamarindus indica* and *Terminalia arjuna* were found to be efficient in the management of kidney stones not only *in vitro* but *in vivo* too.

List of publications

Publications in Peer Reviewed Journals

- Chaudhary A, Singla S K, Tandon C. Calcium oxalate crystal growth inhibition by aqueous extract of *Tamarindus indica*. Indian Journal of Urology. 2008; 24 Suppl 2:S-111. (indexed in PubMed & SCOPUS). (ISSN 0970-1591)

- Chaudhary A, Singla S K, Tandon C. *In vitro* evaluation of *Terminalia arjuna* on calcium phosphate and calcium oxalate crystallization. Indian Journal of Pharmaceutical Sciences (Accepted). (indexed in PubMed)

Communicated

- Chaudhary A, Singla S K, Tandon C. *In vitro* and *in vivo* antilithiatic effect of *Tamarindus indica*: Isolation & purification of a 97kDa antilithiatic protein from it. (Communicated)

- Chaudhary A, Singla S K, Tandon C. Role of an antilithiatic protein inhibitor isolated from *Terminalia arjuna*. (Communicated)

Conferences attended

International conference

- Chaudhary A, Singla S K, Tandon C. Calcium oxalate crystal growth inhibition by aqueous extract of *Tamarindus indica*. 9th Asian Congress of Urology, 3-5th Oct. 2008 New Delhi .

National conference

- Chaudhary A, Singla S K, Tandon C. Anticalcifying protein isolated from *Terminalia arjuna* bark. Symposium on Natural Products in Health and Disease, Biochemical & Molecular Mechanisms, 5-6th March, 2010 Panjab University, Chandigarh.

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