EVALUATION OF *IN-VITRO* AND *IN-VIVO* ANTIUROLITHIATIC ACTIVITY OF VARIOUS EXTRACTS OF WHOLE PLANT OF *Azima tetracantha* Lam. ON ETHYLENE GLYCOL INDUCED UROLITHIASIS IN RATS

A dissertation submitted to

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MASTER OF PHARMACY

IN

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Submitted by

Reg No: 261226053

INSTITUTE OF PHARMACOLOGY

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APRIL – 2013-14

CERTIFICATE

This is to certify that the dissertation entitled **"EVALUATION OF *IN-VITRO* AND *IN-VIVO* ANTIUROLITHIATIC ACTIVITY OF VARIOUS EXTRACTS OF WHOLE PLANT OF *Azima tetracantha* Lam. ON ETHYLENE GLYCOL INDUCED UROLITHIASIS IN RATS"** submitted by **Registration No. 261226053** in partial fulfillment of the requirements for the award of Degree of Master of Pharmacy in Pharmacology by the Tamilnadu Dr. M. G. R. Medical University, Chennai is a bonafide work done by her during the academic year 2013-2014.

The Dean,

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INTRODUCTION

Medicinal plants are major parts of traditional systems in developing countries. Herbal medicine is defined as the branch of science in which plant used formulations are used to alleviate the diseases. It is known as botanical medicine or phytomedicine. Many infectious diseases are known to be treated with herbal remedies throughout the history of mankind. Even today plant materials continue to play a major role in primary health care as therapeutic remedies in many developing countries[1]. Medicinal plants which form the backbone of traditional medicine have in the last few decades have been the subject of very intense pharmacological studies.

Ayurvedha, Siddha, Unani and Folk medications are the main systems of indigenous drugs. Researchers are providing evidence and research, in validating efficacy and safety of utilizing traditional awareness for health and healing.

Medicinal plants are of great economic importance in the Indian subcontinent. The documentation of traditional knowledge especially on the medicinal uses of plants in the history has provided many important drugs of the modern day[2]. 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[3]. Herbal medicines offer conventional treatments, providing safe and well-tolerated remedies for chronic illness which typically resulted from the combinations of secondary plant metabolites that are synthesized and deposited in specific parts or in all parts of the plant. Since, many of the existing synthetic drugs cause various side effects, drugs synthesized from the higher plants continue to occupy an important niche in modern medicine and play an important role in modern medicine and introduction of new therapeutic agents.

1.1 Advantages of Herbal Medicine:

- Herbal medicines have long history of use and better patient tolerance as well as acceptance.
- Medicinal plants have a renewable source, which is our only hope for sustainable supplies of cheaper medicines for the world growing population.
- Availability of medicinal plants is not a problem especially in developing countries like India having rich agro-climatic, cultural and ethnic biodiversity.
- The cultivation and processing of medicinal herbs and herbal products is environmental friendly.
- Prolonged and apparently uneventful use of herbal medicines may offer testimony of their safety and efficacy.

Throughout the world, herbal medicine has provided many of the most potent medicines to the vast arsenal of drugs available to modern medical science, both in crude form and as a pure chemical upon which modern medicines are structured[4].

Urolithiasis is one of the most common diseases of the urinary tract which has been afflicting human kind since antiquity. Urinary stones affect 10-30% of the population in industrialized countries. It occurs more frequently in men than women but rare in children[5]. Urolithiasis is associated with calculus formation at any level in the urinary collecting system, but calculus often arises in the kidney. Recurrent stone formation is probably the most important problem in the after care patients who have undergone operations for renal and ureteric calculi. Urolith formation is a **multifactorial process** which may relate to diet, urinary tract infection, altered urinary solutes and colloids, decreased urinary drainage and urinary stasis, prolonged immobilization, Randall's plaque and microliths etc[6].,

When the urea-splitting organisms infect the urinary tract, bacteria disintegrate the urea excreted in urine in the presence of urease enzyme, which subsequently trigger the formation of ammonia rendering the urine alkaline. In alkaline state, urine leads to contain precipitated crystals of calcium oxalate, magnesium phosphate and calcium carbonate in large amount thereby leading to a strong tendency to form calculi. Bacterial infection may induce stone formation by crystal adherence. Most of the urea-splitting organisms belong to species Proteus but, organisms such as Pseudomonas, Staphylococcus, Escherichia coli and even Mycoplasma were reported to be capable of producing urease[7].

Infected stones were associated with the organisms like E.coli, Proteus species, Streptococcus, Staphylococcus, Pseudomonas and Ureaplasma urealyticum[8]. There are increasing evidence that have been reported that the end products of urealysis damage the glycosaminoglycon layer of the renal urolithial cells thus leading to the bacterial adherence, biofilm formation and mineral encrustation. Exhaustive microbiological investigations are therefore necessary to diagnose and treat the infection responsible for the stone formation.

Urinary tract stone disease has been documented historically as far back as Egyptian mummies[9.] The experimental intoxication induced by ethylene glycol is widely used for kidney stone formation in rats. When ethylene glycol is metabolized by the body, it produces **toxic metabolites like glycoaldehyde, glycolate and glyoxylate**. These metabolites cause tissue destruction, primarily from calcium oxalate deposition and metabolic abnormalities, specifically a high anion-gap metabolic acidosis, lactic acidosis and hypocalcemia. Oxalic acid combines with calcium to form calcium oxalate crystals, which deposit in the kidneys. **This can result in hematuria and proteinuria, increased creatinine and renal failure[10]**.

Surgical operation, lithotripsy and local calculus disruption using high power laser are widely used to remove the calculi. Many remedies have been employed since ages to treat

renal stones and most of them were from plants and proved to be useful. In Ayurvedha and Folklore medicine many herbs are used in management of urolithiasis[11].

Azima tetracantha Lam. (Family: Salvadoraceae) locally known as "Mulsangu", is a scrambling spinous shrub flowering throughout the year found in Peninsular India, West Bengal, Orissa, African countries and extends through Arabia to tropical Asia. The leaves of the plant are elliptical in shape and are rigid, pale green coloured. The flowers are small, greenish white (or) yellow coloured, unisexual in axillary fasciles. The berries are white in colour, usually one seeded and edible. The plant is considered as a powerful diuretic and also used to treat rheumatism, dropsy, dyspepsia, chronic diarrhea and as a stimulant tonic for women after confinement.

It also possesses anti-inflammatory, anti-pyretic and analgesic activity. The plant shows potent antibacterial and antifungal activity. However, till the date there have been no investigations supporting the anti-urolithiatic properties of this plant. Hence, this study has been taken with an aim to evaluate the anti-urolithiatic potential of *Azima tetracantha* Lam. extract in *in-vivo* ethylene glycol model.

AIM & OBJECTIVE

- To identify the active phytochemical components in the whole plant extract of *Azima tetracanth*
- To determine the Calcium oxalate crystallization inhibition by *in-vitro* method.
- To investigate the *in-vivo* anti urolithiatic effect of various extract of *Azima tetracantha* Lam. in Ethylene glycol induced urolithiasis in Wistar albino rats.

REVIEW OF LITERATURE

3.1 PLANT PROFILE

Plant name: *Azima tetracantha* Lam.

Synonym: Monetia barlerioides

TAXONOMY

Kingdom: Plantae

Division: Magnoliophyta

Class: Magnoliopsida

Sub-class: Dilleniidae

Order: Brassicales

Family: Solvadoraceae

Genus: Azima

Species: tetracantha

VERNACULAR NAMES[12]

Tamil: Mulsangu

Kannada: Uppimullu

Ayurvedic medicine: Kundali

Malayalam: Esanku

Azima tetracantha Lam.

English: Needle bush

DISTRIBUTION

Azima tetracantha Lam. is a scrambling spinous shrub or small tree of about 5 m flowering throughout the year found in Peninsular India, West Bengal, Orissa, African countries and extends through Arabia to tropical Asia.

PARTS USED

Whole plant

DESCRIPTION

Stem: Bark green on younger branches, turning brown, young twigs sometimes square in cross-section, hairy; characteristic whorls of four long straight spines occur along the length of branches at each of the leaf axils.

Leaves: Oval to circular, opposite or nearly so, each pair at right angles to the previous and following one; light green, leathergy and usually hairy; apex has a sharp tip, margin entire, tapering at both ends, short petiole.

Flowers: Dioecious; light green or yellow, small flower clusters in axils; floral parts in fours; petals recurving, calyx bell-shaped.

Seed/Fruit: Round berry of 1cm diameter with a sharp apical tip; fleshy, light colored, containing one or two seeds; ripe from summer into the next winter.

CHEMICAL CONSTITUENTS[13,14,15]

The plant contains alkaloids, flavonoids, sterols, terpenoids, volatile oil and fatty acids, saponins.

A number of chemical constituents have been reported from the leaves (Friedelin, Lupeol, Glutinol and beta-sitosterol), the seed oils (fatty acids such as myristic acid, palmitic acid, stearic acid, arachidic acid, oleic acid, linoleic acid, eicosenoic acid and flavonoids), the seed, root, stem and young leaves (N-methoxy-3-indolyl methyl-glucosinolate), roots and the leaves (terpenoids) also the dimeric pipperidine alkaloids azimine, azcarpine and carpaine are present in all plant parts.

MEDICINAL USES

- The juice of the leaves is used to relieve the cough phthisis and asthma.
- In western India juice of the leaves is applied as eardrops against ear ache and crushed leaves placed on painful teeth.
- In India and Sri Lanka the root, root bark and leaves are administered with food as a remedy for rheumatism.
- The plant is considered as a powerful diuretic and also used to treat dropsy, dyspepsia, chronic diarrhea and as a stimulant tonic for women after confinement.
- The leaves of this plant possess anti-inflammatory, anti-ulcer and analgesic[16] activity.
- This plant possesses anti-bacterial and anti-fungal properties.

3.2 PLANT REVIEW:

Antimicrobial activity

Gayathri G *et al.,* in 2011 revealed that methanolic extract of *Azima tetracantha* Lam. leaves possess greater antimicrobial activity than chloroform extract. The test extracts showed percent inhibition in a concentration dependent manner against the test organisms such as S.aureus, P.vulgaris etc[17].

Hema *et al*., in 2012 revealed that the antimicrobial activity of leaves of the medicinal plant *Azima tetracantha* Lam. collected from the regions of Ambalathara, Kerala, South India was checked against the clinical pathogens by agar well diffusion method. *Azima tetracantha* Lam. showed highest antimicrobial activity on ethanolic extracts. Antimicrobial activities of five solvent extracts (ethanol, methanol, acetone, chloroform and distilled water) were tested against seven clinical pathogens such as Staphylococcus aureus (Pus), Klebsiella sp. (Sputum), Escherichia coli (Urine), Serratia sp. (Sputum) and Proteus sp. (Sputum). Among the five solvents tested, ethanolic extracts of *Azima tetracantha* Lam. showed higher significant activity against the pathogenic organisms[18].

Antibacterial activity of *Azima tetracantha* Lam. and phytochemicals separated from *Azima tetracantha* Lam. were alkaloids, flavanoids and sterol and were tested against Staphylococcus aureus, Bacillus subtiles, Klebsiella pneumonia, Pseudomonas aeruginosa and E.coli (**Gowthami *et al*., 2012**). The sterols compound exhibited maximum zone of inhibition against Bacillus subtilis (25mm) and Pseudomonas aeruginosa (24mm). The alkaloid compound showed minimum zone of inhibition was observed in Bacillus subtilis (12mm) and Pseudomonas aeruginosa (12mm). Maximum zone of inhibition was observed in sterols compound when compared with alkaloids and flavonoids[19].

The study designed by **Duraipandyan *et al.,* in 2011** evaluated the antifungal activity of *Azima tetracantha* Lam. Extracts and isolated compound (friedelin) against fungi.

Antifungal activity was carried out using broth microdilution method and fractions were collected using (silica gel) column chromatography. The antifungal activity of *Azima tetracantha* Lam. crude extracts and isolated compound (friedelin) were evaluated using the microdilution method. Hexane extract showed some antifungal activity. The results suggested that Friedelin is a strong antifungal agent[20].

Anti-inflammatory, analgesic and antipyretic effects of friedelin isolated from *Azima tetracantha Lam.*

Paulrayer *et al*., in 2011 revealed the effects of friedelin on inflammation were studied by using carrageenan-induced hind paw oedema, croton oil-induced ear oedema, acetic acid-induced vascular permeability, cotton pellet-induced granuloma and adjuant-induced arthritis. The analgesic effect of friedelin was evaluated using the acetic acid-induced paw liking response and the hot-plate test. The antipyretic effect of friedelin was evaluated using the yeast-induced hyperthermia test in rats.

In the acute phase of inflammation, maximum inhibitions were noted with friedelin in carrageenan-induced ear oedema. Administration of friedelin significantly decreased the formation of granuloma tissue. Friedelin also produced significant analgesic activity in the acetic acid-induced abdominal constriction response and formalin-induced paw licking response. Treatment with friedelin showed a significant dose-dependent reduction in pyrexia in rats[21].

The results of the study made by **Nargis Begum T *et al*., in 2011** suggest that the ethanolic leaf extract of *Azima tetracantha* Lam. in doses of 100 and 200 mg/kg, significantly reduce the temperature of pyretic rats as revealed from the observation that the average percentage of antipyretic activity increased with the concentration of the extracts (200 mg/kg) compared with the control[22].

Antioxidant and free radical scavenging activities of *Azima tetracantha Lam*. leaf extracts

Yildrim *et el.,* in 2000 confers the antioxidant effect of *Azima tetracantha* Lam. leaf extracts. Phenolic compounds are typical active oxygen scavengers in plants and are known to contribute directly to antioxidant action. The results indicate a high concentration of polyphenols in the leaves of *Azima tetracantha* Lam.. The hydroxyl groups of the phenolic compounds confer the scavenging ability of the plant. The decrease in absorbance of DPPH radical is due to its reduction by different antioxidants, which in turn indicates the free radical scavenging property of the leaves of *Azima tetracantha* Lam[23].

The studies conducted by **Siriwardhana *et al.,* (2003**) reported a high correlation between DPPH radical scavenging potential and total phenolic content[24].

The reducing capacity of a compound may also serve as a significant indicator of its potential antioxidant activity[25] (**Sreekanth *et al.,* 2003, Leskovar *et al.,* 2004**).

In a study done by **Gayathri G *et al*., in 2011**, the reducing capacity increased with increasing concentration of the plant extract. This shows that the antioxidant compounds can react with increasing concentration of the plant extract. This shows that the antioxidant compounds can react with free radical to convert them to more stable products and thereby terminate radical chain reactions[17].

Thendral et al., in 2010 revealed that *Azima tetracantha* Lam. leaves were proved to be good source of natural phenolic compounds. The methanolic extract of the leaves showed better free radical capacity against different reactive oxygen/nitrogen species, among other extracts although with different efficiencies. The high content of antioxidants like phenolic compounds, flavonoids and vitamins found in these extracts, may impart health benefits by combating the free radicals in synergistic manner along with other compounds and thus constitute part of the basis for the ethno pharmacological claim[26].

According to **Maruthi T Ekbote et al., in 2010,** the ethanolic extract of *Azima tetracantha* leaves exhibited a significant antioxidant effect showing increased levels of enzymatic and non-enzymatic parameters, viz. catalase, GSH, total thiols and decreased level of malondialdehyde[30].

Antinephrotoxic potential of *Azima tetracantha* Lam

The biochemical markers of nephrotoxicity are urea, creatinine and GGT. Their levels are significantly elevated in nephrotoxic condition due to metal induced damage to nephrons. In nephrotoxicity, the serum urea and creatinine accumulates because the rate of serum urea and creatinine production exceeds the rate of clearance due to defects in the glomerular filtration rate. The results of the study done by **Manikandanselvi *et al*., in 2012** shows the significant elevation in the levels of urea, GGT and creatinine in ferrous sulphate induced group compared to control. After treatment with herbal drug viz*, Azima tetracantha Lam*. There was a significant decrease in the levels near to normal compared to ferrous sulphate induced group[27].

Antiulcer activity of *Azima tetracantha* Lam

Muthusamy *et al.,* in 2009 reveals that ethanolic extract of *Azima tetracantha Lam.* showed significant dose-dependent ulcer protective effect against cold restraint stress and aspirin plus pylorus ligation induced gastric ulcers. The gastro duodenal ulcer protecting effect of ethanolic extract of *Azima tetracantha* Lam. may be due to its predominant effect on the mucosal defensive factors rather than offensive factors[28].

Hepatoprotective activities of *Azima tetracantha* Lam

Reports documented by **Nargis *et al*.,in 2011** reveals that the rats treated with ethanolic extract of *Azima tetracantha* Lam. showed a significant reduction in all the five-biochemical parameters of liver damage (AST, ALT,ALP,ACP and total bilirubin) elevated by carbon tetrachloride. Ethanolic leaf extract treatment of *Azima tetracantha* Lam, showed

more significant reduction of AST, ALT, ACP and total bilirubin[32].

Results recorded by **Arthika *et al*., in 2011** shows that the ethanolic extract of *Azima tetracantha* reduced the hepatotoxin intoxication induced elevated biochemical parameters and decrease theprotein synthesis and accumulation of triglycerides leading to fatty liver. Reduction of raised bilirubin level suggests the stability of the biliary function during the hepatic injury with paracetamol. Treatment with ethanolic extract of *Azima tetracantha* significantly reversed it, indicating that the phytoconstituents present in this extraction have hepatoprotective potential[29].

A study done by **Maruthy T Ekbote *et el*., in 2010** provides scientific evidence on the correlative effects of hepatoprotective activities of *Azima tetracantha* Lam. They induced hepatotoxicity by altering liver microsomal membranes in experimental animals by CCl_4 administration. The chloroform and ethanol extracts of *Azima tetracantha* Lam. reduced the hepatotoxin intoxication induced elevated biochemical parameters and decrease the protein synthesis[30].

Balakrishnan *et al*., in 2012 reported that in the liver sections of the rats treated with ethanol extract of *Azima tetracantha* Lam. root bark extract for 7 days, the normal cellular architecture was retained there by further confirming the potent hepatoprotective effect of ethanolic extract of *Azima tetracantha* Lam. root bark. The ethanol (50%) extract of *Azima tetracantha* Lam. (EEAT) root bark afforded significant protection against CCl_4 induced hepatocellular injury[31].

Hypoglycemic and antihyperlipidemic activity of *Azima tetracantha* Lam.

The ethanolic leaf extract of *Azima tetracantha* Lam. was investigated by **Nargis *et al*., in 2009** for hypoglycemic and hypolipidemic activity in alloxan-induced diabetic albino rats. The ethanolic leaf extract of *Azima tetracantha* Lam. produced significant reduction in plasma glucose and also had beneficial effects on the lipid profile in alloxan-induced diabetic

rats at the end of the treatment period[32].

ACUTE TOXICITY STUDIES:

Muthusamy*et al.,*2009 showed that the extract of *Azima tetracantha* Lam. possess antiulcer activity with LD_{50} of 2000 mg/kg[28].

3.3 UROLITHIASIS

Urolithiasis (from Greek ouron, "urine" and lithos, "stone") is the condition where urinary stones are formed or located anywhere in the urinary system. The term nephrolithiasis (or "renal calculus") refers to stones that are in the kidney, while ureterolithiasis refers to stones that are in the ureter. The term cystolithiasis (or vesical calculi) refers to stones which form or have passed into the urinary bladder[33].

Urinary stones are typically classified by their location or by their chemical composition. In humans, calcium oxalate is a major constituent of most urinary stones. About 80% of those with kidney stones are men. Men most commonly experience their first episode between 20-30 years of age, while for women the age at first presentation is somewhat later.

Nomenclature of Stones:

The word "crystal "is derived from the Greek work krystallosus, which means " ice " and is used to refer to the solid phase of substances having a specific internal structure and enclosed by symmetrically arranged planar surfaces.

The Latin word *calculus* means "pebble". The crystalline constituents of urinary calculi in the human are varied. Some of these occur geologically, whereas others are found only in the animal kingdom[34].

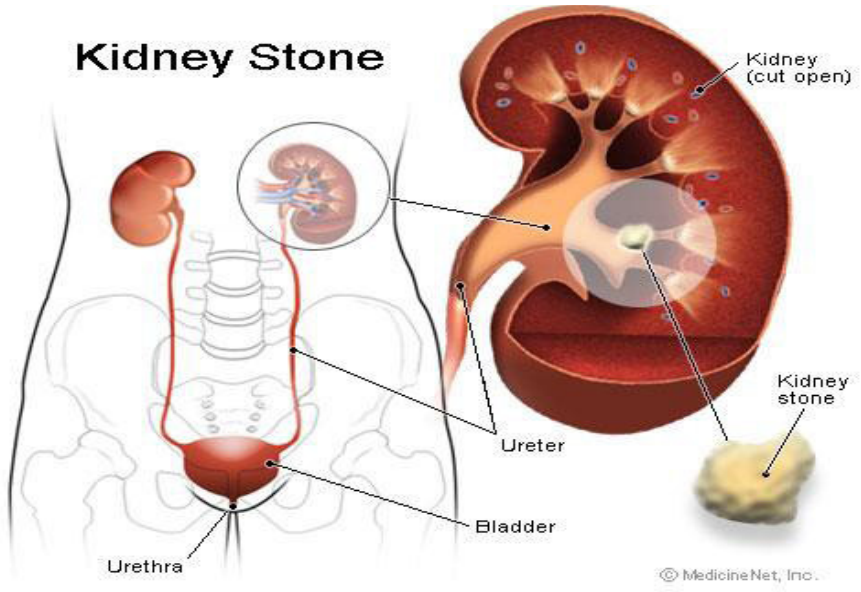
Stones can form when urine contains too much of certain substances. These substances can create small crystals that become stones. The stones take weeks or months to form. There are different types of kidney stones. The exact cause depends on the type of stone[35].

Nephrolithiasis and ureterolithiasis

A kidney stone, also known as a renal calculuoos is a solid **concretion** or **crystal** aggregation formed in the kidneys from **dietary minerals** in the urine. Kidney stones typically leave the body by passage in the urine stream, and many stones are formed and passed without causing symptoms. If stones grow to sufficient size (usually at least 3millimeters (0.12n)) they can cause obstruction of the ureter. Ureteral obstruction causes **postrenal azotemia** and **hydronephrosis** (distension and dilation of the **renal pelvis** and **calyces**), as well as spasm of the ureter. This leads to pain, most commonly felt in the flank (the area between the ribs and hip), lower abdomen and groin (a condition called renal colic). Renal colic can be associated with nausea, vomiting, fever, blood in the urine, pus in the urine and painful urination. Renal colic typically comes in waves lasting 20 to 60 minutes, beginning in the flank or lower back and often radiating to the groin or genitals.

Etiology and Precipitating Factors:

- Urinary tract infection (UTI) which increases the alkalinity of the urine and causes calcium and other substance to precipitate and form renal calculi.
- Immobility, dehydration and urinary obstruction or stasis, increasing likelihood that calculus forming substances well precipitate.
- Metabolic or dietary changes, such as hyperthyroidism, hyperparathyroidism, bone disease, corticosteroid use, excessive vitamin A and D intake, diet high in calcium or purine, or other factors increasing calcium, phosphorus, uric acid and other calculus-forming substances in the blood or urine.
- More common in male aged 30-50 years[37].



Types of kidney stones:

TYPES OF KIDNEY STONES

Urinary stones are typically classified by their location in the kidney (nephrolithiasis), or bladder (cystolithiasis), or ureter (ureterolithiasis), or chemical composition (calcium-containing, struvite, uric acid or other compounds).

Table 3.1 Types of Kidney stones

Kidney Stone type	Population	Circumstances	Details
Calcium Oxalate	80%	when urine is alkaline (ph>5.5)	Some of the oxalate in urine is produced by the body. Calcium and oxalate in the diet play a part but are not the only factors that affect the formation of calcium oxalate stones. Dietary oxalate is an organic molecule found in many vegetables, fruits, and nuts. Calcium from bone may also play a role in kidney stone formation.
Calcium phosphate	—	when urine is alkaline (high pH)	
Uric acid	5-10%	when urine is persistently acidic	Diets rich in animal proteins and purines: substances found naturally in all food but especially in organ meats, fish, and shellfish.
Struvite	10-15%	infections in the kidney	Preventing struvite stones depends on staying infection-free. Diet has not been shown to affect struvite stone formation.
Cystine	—	rare genetic disorder	Cystine, an amino acid (one of the building blocks of protein), leaks through the kidneys and into the urine to form crystals.

Calcium stones are most common. They are most common in men between the ages of 20 and 30. Calcium can combine with other substances, such as oxalate (the most common substance), phosphate or carbonate to form the stone. Oxalate is present in certain foods such

as spinach. It is also found in vitamin C supplements. Diseases of the small intestine increase risk of bone disease.

Cystine stones can form in people who have cystinuria. This disorder runs in families and affects both men and women.

Struvite stones are mostly found in women who have a urinary tract infection. These stones can grow very large and can block the kidney, ureter or bladder.

Uric acid stones are more common in men than in women. They can occur with gout or chemotherapy[37].

Urine calcium >300mg/24h (men), 250mg/24h (women), or 4mg/kg per 24h either sex. Hyperthyroidism, Cushing's syndrome, sarcoidosis, malignant tumour, immobilization, vitamin D intoxication, rapidly progressive bone disease and Paget's disease all cause hypercalciuria and must be excluded in diagnosis of idiopathic hypercalciuria.

Table 3.2 Symptoms of urolithiasis based on stone location

Stone location	Common symptoms
Kidney	Vague flank pain, hematuria
Proximal ureter	Renal colic, flank pain, upper abdominal pain
Middle section of ureter	Renal colic, anterior abdominal pain, flank pain
Distal ureter	Renal colic, dysuria, urinary frequency, anterior abdominal pain, flank pain

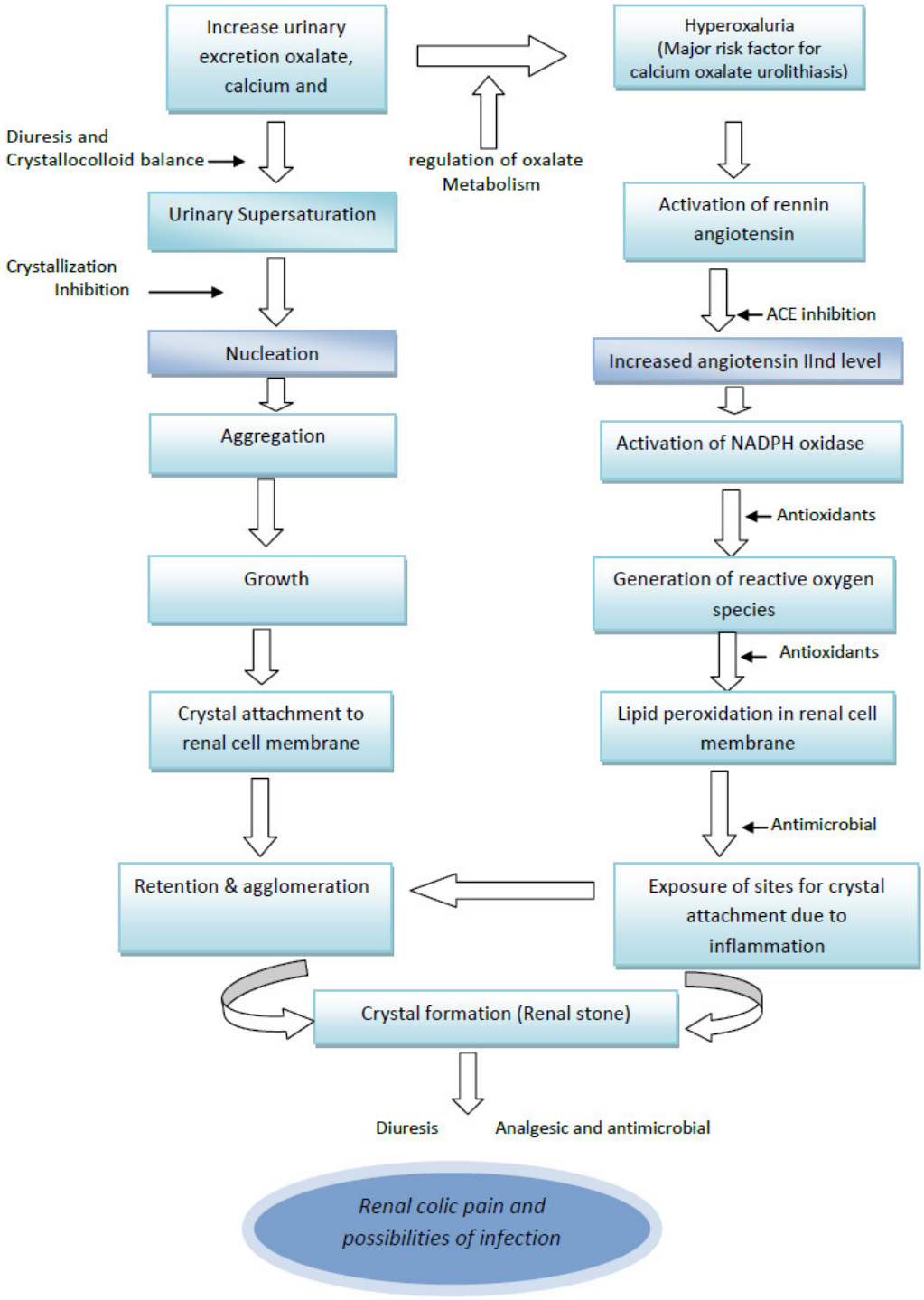


Fig 3.1 Mechanism of Urolithiasis[38]

MANIFESTATIONS OF STONE[36]

As stones grow on the surfaces of the renal papillae or within the collecting system, they do not necessarily produce symptoms. Asymptomatic stones may be discovered during the course of radiographic studies undertaken for unrelated reasons. Stones are a common cause of isolated hematuria. Stones become symptomatic when they enter the ureter or occlude the ureteropelvic junction, causing pain and obstruction.

Stone passage

A stone can traverse the ureter without symptoms, but passage usually produces pain and bleeding. The pain begins gradually, usually in the flank, but increases over the next 20-60min to become so severe that narcotics may be needed for its control. The pain may remain in the flank or spread downward and anteriorly toward the ipsilateral loin, testis or vulva. A stone in the portion of the ureter within the bladder wall causes frequency, urgency and dysuria that may be confused with urinary tract infection. The vast majority of ureteral stones <0.5cm in diameter pass spontaneously.

There may not be any symptoms until the stones move down the ureters through which urine empties into the bladder. When this happens, the stones can block the flow of urine out of the kidneys[36].

The main symptom is severe pain that starts suddenly and may go away suddenly:

- Pain may be felt in the belly area or side of the back.
- Pain may move to groin area (groin pain) or testicle (testicle pain).

Other symptoms can include:

- Abnormal urine color
- Hematuria (Blood in urine)

- Pyuria (Pus in urine)
- Dysuria (Burning on urination when passing stones)
- Oliguria (Reduced urinary volume)
- Chills
- Fever
- Nausea, vomiting[39]

Potential Complications

- Bleeding (may be acute or delayed for 1 to 2 weeks).
- Sepsis
- Renal pelvis perforation and loss of irrigating fluid into retroperitoneal area
- Non removable calculi
- Loss of calculus fragments into retro peritoneum[40].

CAUSES

Dietary factors that increase the risk of stone formation include

- Low fluid intake
- High dietary intake of animal protein
- Sodium
- High fructose corn syrup
- Oxalate
- Grapefruit juice
- Apple juice
- Cola drinks

The biggest risk factor for kidney stone is not drinking enough fluids. Kidney stones are more likely to occur if less than 1 liter of urine is passed in a day[33].

Medications Increasing the Tendency for Stone Formation

- ✓ Acetazolamide (calcium phosphate stones)
- ✓ Allopurinol (xanthine or oxypurinol stones)
- ✓ Calcium carbonate
- ✓ Chemotherapy (increased urate load)
- ✓ Ephedra/guaifenesin
- ✓ Indinavir/acyclovir
- ✓ Sulfonamides
- ✓ Triamterene
- ✓ Vitamine C (oxalate)
- ✓ Vitamine D, nonthiazide diuretic, steroids (hypercalciuria)[41]

Diagnostic studies

Urine analysis: Commonly shows red blood cells, white blood cells, crystals, minerals and pH changes.

Urine culture: Commonly shows presence of bacteria.

24 hours urine study: Commonly shows high levels of calcium, phosphorus, uric acid, creatinine, oxalate or cystine.

Renal calculus analysis: Shows mineral composition of stones.

Blood Studies: May show high serum level of calcium, protein, electrolyte, uric acid, phosphates, blood urea nitrogen, creatinine or WBCs[40].

TESTS

- Noncontrast helical CT has become the standard for evaluation of acute urolithiasis.

- Renal/Bladder ultrasound; most useful screening test.
- Abdominal plain radiography may show calcifications; uric acid calculi are radiolucent.
- Intravenous excretory urography allows imaging of renal parenchyma, collecting system and ureters.
- Stone component identification[41].

TREATMENT

Treatment depends on the type of stone and the severity of the symptoms.

Common treatments are,

- Kidney stones that are small usually pass on their own. When the stone passes, the urine should be strained so the stone can be saved and tested.
- Drinking at least 6-8 glasses of water per day to produce a large amount of urine is recommended.
- Pain can be severe enough to need narcotic pain relievers.

Calcium Stones[36]

Idiopathic Hypercalciuria

This condition is the most common metabolic abnormality found in patients with nephrolithiasis. It is familial and is probably a polygenic trait, although there are some rare monogenic causes of hypercalciuria and kidney stones such as Dent's disease, which is an X-linked disorder characterized by hypercalciuria, nephrocalcinosis, and progressive kidney failure. Idiopathic hypercalciuria is diagnosed by the presence of hypercalciuria without hypercalcemia and the absence of other systemic disorders known to affect mineral metabolism. Vitamin D overactivity through either high calcitriol levels or excess vitamin D

receptor is a likely explanation for the hypercalciuria in many patients. Recent studies have shown that a polymorphism (Arg990Gly) of the calcium-sensing receptor, which leads to activation of the receptor, is more common in hypercalciuric subjects and probably contributes to higher urine calcium excretion. Hypercalciuria contributes to stone formation by raising urine saturation with respect to calcium oxalate and calcium phosphate.

Treatment: Hypercalciuria

For many years the standard therapy for hypercalciuria was dietary calcium restriction. However, studies have shown that low-calcium diets increase the risk of incident stone formation, perhaps by reducing the amount of calcium in the intestine to bind oxalate, thereby increasing urine oxalate levels. A 5-year prospective trial compared the efficacy of a low-calcium diet to a low-protein, low-sodium and normal-calcium diet in preventing stone recurrence in male calcium stone formers. The group on the low-calcium diet had a significantly greater rate of stone relapse. In addition, hypercalciuric stone formers have reduced bone mineral density and an increased risk of fracture compared with the non-stone-forming population. Low calcium intake probably contributes to the low bone mineral density. In sum, low-calcium diets are of unknown efficacy in preventing stone formation and carry a long-term risk of bone disease, making low-sodium and low-protein diets a superior treatment option.

If diet therapy is not sufficient to prevent stones, thiazide diuretics may be used. Thiazide diuretics lower urine calcium and are effective in preventing the formation of stones. Three year randomized trials have shown a 50% decrease in stone formation in the thiazide-treated groups compared with the placebo-treated controls. The drug effect requires slight contraction of the extracellular fluid volume and high dietary NaCl intake reduces its

therapeutic effect. Thiazide-induced hypokalemia should be treated aggressively since hypokalemia will reduce urine citrate, an important inhibitor of calcium crystallization.

Hyperuricosuria

About 20% of calcium oxalate stone formers are hyperuricosuric, primarily because of an excessive intake of purine from meat and fish. The mechanism of stone formation probably involves salting out calcium oxalate by urate. A low-purine diet is desirable but difficult for many patients to achieve. The alternative is allopurinol, which has been shown to be effective in a randomized, controlled trial.

Hyperoxaluria

Oxalate is a metabolic end product in humans. Urine oxalate comes from diet and endogenous metabolic production, with ~40–50% originating from dietary sources. The upper limit of normal for oxalate excretion is generally considered to be 40–50mg per day. Mild hyperoxaluria (50–80mg/d) usually is caused by excessive intake of high-oxalate foods such as spinach, nuts and chocolate. In addition, low-calcium diets may promote hyperoxaluria as there is less calcium available to bind oxalate in the intestine. Enteric hyperoxaluria is a consequence of small-bowel disease, resulting in fat malabsorption. Oxalate excretion is often >100mg per day. Enteric hyperoxaluria may be caused by jejunoileal by pass for obesity, pancreatic insufficiency, or extensive small-intestine involvement from Crohn's disease. With fat malabsorption, calcium in the bowel lumen is bound by fatty acids instead of oxalate, which is left free for absorption in the colon. Delivery of unabsorbed fatty acids and bile salts to the colon injures the colonic mucosa and enhances oxalate absorption. Recent studies have shown that modern bariatric surgery for obesity that involves by passing intestinal segments, such as Roux-en-Y gastric bypass and

biliopancreatic diversions, may lead to hyperoxaluria that can cause kidney failure as well as kidney stones. The mechanism of hyperoxaluria has not been well studied.

Primary hyperoxaluria is a rare autosomal recessive disease that causes severe hyperoxaluria. Patients usually present with recurrent calcium oxalate stones during childhood. Primary hyperoxaluria type 1 is due to a deficiency in the peroxisomal enzyme alanine: glyoxylate aminotransferase. Type 2 is due to a deficiency of D-glyceric dehydrogenase. Severe hyperoxaluria from any cause can lead to stone formation and produce tubulointerstitial nephropathy.

Treatment: Hyperoxaluria

Patients with mild to moderate hyperoxaluria should be treated with a diet low in oxalate and with a normal intake of calcium and magnesium to reduce oxalate absorption. Enteric hyperoxaluria can be treated with a low-fat, low-oxalate diet and calcium supplements, given with meals, to bind oxalate in the gut lumen. The oxalate-binding resin cholestyramine provides an additional form of therapy. Treatment for primary hyperoxaluria includes a high fluid intake, neutral phosphate, potassium citrate and pyridoxine (25–200mg/d). Even with aggressive therapy, irreversible renal failure may occur. Liver transplantation to correct the enzyme defect, combined with kidney transplantation, has been successfully utilized in patients with primary hyperoxaluria.

Hypocitraturia

Urine citrate prevents calcium stone formation by creating a soluble complex with calcium, effectively reducing free urine calcium. Hypocitraturia is found in 20–40% of stone formers either as a single disorder or in combination with other metabolic abnormalities. It can be secondary to systemic disorders such as RTA, chronic diarrhoeal illness and

hypokalemia or it may be a primary disorder, in which case it is called *idiopathic hypocitraturia*.

Treatment: Hypocitraturia

Treatment is with alkali, which increases urine citrate excretion; generally, bicarbonate or citrate salts are used. Potassium salts are preferred as sodium loading increases urinary excretion of calcium, reducing the effectiveness of treatment. Two randomized, placebo-controlled trials have demonstrated the effectiveness of citrate supplements in calcium oxalate stone formers. Lemonade and other citrate-rich beverages have been used to treat hypocitraturia, although the increase in urine citrate is not as great as is seen with pharmacologic dosing of citrate salts.

Idiopathic Calcium Lithiasis

Some patients have no metabolic cause for stones despite a thorough metabolic evaluation. The best treatment appears to be high fluid intake so that the urine specific gravity remains at 1.005 throughout the day and night. Thiazide diuretics and citrate therapy may help to reduce crystallization of calcium salts, but there have been no prospective trials in this patient population. Oral phosphate at a dose of 2g phosphorus daily may lower urine calcium and increase urine pyrophosphate, thereby reducing the rate of recurrence. Orthophosphate causes mild nausea and diarrhea, but tolerance may improve with continued intake.

Uric Acid Stones[36]

Persistently acidic urine is the major risk factor for uric acid stone formation. When urine pH is low, the protonated form of uric acid predominates and is soluble in urine at concentrations of 100mg/L. Concentrations above this level represent supersaturation that causes crystals and stones to form. Common causes of acidic urine and uric acid stones include metabolic syndrome, chronic diarrhoeal states, gout and idiopathic uric acid lithiasis.

As the prevalence of obesity increases, metabolic syndrome is becoming an increasingly important cause of uric acid stone formation, as insulin resistance leads to a decrease in ammoniagenesis, requiring that the metabolic acid load be excreted as titratable acid. Hyperuricosuria, when present, increases super saturation, but urine of low pH can be supersaturated with uric acid even though the daily excretion rate is normal. Myeloproliferative syndromes, chemotherapy for malignant tumors and Lesch-Nyhan syndrome cause such massive production of uric acid and consequent hyperuricosuria that stones and uric acid sludge form even at a normal urine pH. Obstruction of the renal tubules by uric acid crystals can cause acute renal failure.

Treatment: Uric Acid Lithiasis

The two goals of treatment are to raise urine pH and lower excessive urine uric acid excretion to <1g/d. Supplemental alkali, 1–3 mEq/kg of body weight per day, should be given in three or four divided doses, one of which should be given at bedtime. The goal of treatment should be a urine pH between 6 and 6.5 in a 24-h urine collection. Increasing urine pH above 6.5 will not provide additional benefit in preventing uric acid crystallization but increases the risk of calcium phosphate stone formation. The form of the alkali may be important. Potassium citrate may reduce the risk of calcium salts crystallizing when urine pH is increased, whereas sodium alkali salts may increase the risk. A low-purine diet should be instituted in uric acid stone formers with hyperuricosuria. Patients who continue to form uric acid stones despite treatment with fluids, alkali and low-purine diet.

Cystinuria and Cystine Stones

In this inherited disorder, proximal tubular and jejunal transport of the dibasic amino acids cystine, lysine, arginine, and ornithine is defective, and excessive amounts are lost in

the urine. Clinical disease is due solely to the insolubility of cystine. Cystine crystals plug terminal collecting ducts, and stones may grow as an extension of those plugs. Damage to the papillae and medulla from crystal obstruction is the probable reason why kidney function is reduced in cystinuria compared with routine stone disease.

Pathogenesis

Cystinuria occurs because of defective transport of dibasic amino acids by the brush borders of renal tubule and intestinal epithelial cells. Disease-causing mutations have been identified in both the heavy and light chains of a heteromeric amino acid transporter found in the proximal tubule of the kidney. Cystinuria is classified into two main types, based on the urinary excretion of cystine in obligate heterozygotes. In type I cystinuria, heterozygotes have normal urine cystine excretion; thus, type I has an autosomal recessive pattern of inheritance. A gene on chromosome 2 that has been designated *SLC3A1* encodes the heavy chain of the transporter and has been found to be abnormal in type I. In non-type I cystinuria, heterozygotes have moderately elevated urine cystine excretion, with homozygotes having a much higher urine cystine excretion. Non-type I is inherited as a dominant trait with incomplete penetrance. Non-type I is due to mutations in the *SLC7A9* gene on chromosome 19, which encodes the light chain of the heteromeric transporter. In rare cases, mutations of the *SLC7A9* gene can lead to a type I phenotype.

Diagnosis

Cystine stones are formed only by patients with cystinuria, but 10% of stones in cystinuric patients do not contain cystine; therefore, every stone former should be screened for the disease. The sediment from a first morning urine specimen in many patients with homozygous cystinuria reveals typical hexagonal, platelike cystine crystals. Cystinuria can also be detected by using the urine sodium nitroprusside test. Because the test is sensitive, it

is positive for cystinuria in many asymptomatic heterozygotes. A positive nitroprusside test or the finding of cystine crystals in the urine sediment should be evaluated by measurement of daily cystine excretion. Cystine stones seldom form in adults unless urine excretion is at least 300mg/d.

Treatment: Cystinuria and Cystine Stones

High fluid intake, even at night, is the cornerstone of therapy. Daily urine volume should exceed 3L. Raising urine pH with alkali is helpful provided that the urine pH exceeds 7.5. A low-salt diet (100mmol/d) can reduce cystine excretion up to 40%. Because side effects are common, drugs such as penicillamine and tiopronin, which form mixed soluble disulfide cysteine-drug complexes, should be used only when fluid loading, salt reduction and alkali therapy are ineffective. Low-methionine diets have not proved to be practical for clinical use, but patients should avoid protein gluttony.

Struvite Stones

These stones are a result of urinary infection with bacteria, usually *Proteus* species, which possess urease, an enzyme that degrades urea to NH_3 and CO_2. The NH_3 hydrolyzes to NH_4^+ and raises urine pH to 8 or 9. The NH_4^+ precipitates PO_4^{3-} and Mg^{2+} to form $MgNH_4PO_4$ (struvite). Struvite does not form in urine in the absence of infection, because NH_4^+ concentration is low in urine that is alkaline in response to physiologic stimuli. Chronic *Proteus* infection can occur because of impaired urinary drainage, urologic instrumentation or surgery and especially with chronic antibiotic treatment, which can favor the dominance of *Proteus* in the urinary tract. The presence of struvite crystals in urine, rectangular prisms that resemble coffin lids, indicates infection with urease-producing organisms.

Treatment: Struvite Stones

Complete removal of the stone with subsequent sterilization of the urinary tract is the treatment of choice for patients who can tolerate the procedures. Percutaneous nephrolithotomy is the preferred surgical approach for most patients. At times, extracorporeal lithotripsy may be used in combination with a percutaneous approach. Open surgery is rarely required. Irrigation of the renal pelvis and calyces with hemiacidrin, a solution that dissolves struvite, can reduce recurrence after surgery. Stone-free rates of 50–90% have been reported after surgical intervention. Antimicrobial treatment is best reserved for dealing with acute infection and for maintenance of sterile urine after surgery. Urine cultures and culture of stone fragments removed at surgery should guide the choice of antibiotic. For patients who are not candidates for surgical removal of a stone, acetohydroxamic acid, an inhibitor of urease, can be used. Unfortunately, acetohydroxamic acid has many side effects, such as headache, tremor and thrombophlebitis that limit its use.

Surgical options available are:

- Extracorporeal shock-wave lithotripsy is used to remove stones slightly smaller than a half an inch that are located near the kidney or ureter. It uses sound or shock waves to break up stones. Then, the stones leave the body in the urine.
- Percutaneous nephrolithotomy is used for large stones in or near the kidney, or when the kidneys or surrounding areas are incorrectly formed. The stone is removed with tube (endoscope) that is inserted into the kidney through a small surgical cut.
- Ureteroscopy may be used for stones in the lower urinary tract.
- Rarely, open surgery (nephrolithotomy) may be needed if other methods do not work or are not possible[37].

Herbal Home Remedies for Kidney Stones:

- ✓ Kidney beans
- ✓ Watermelon
- ✓ Apple
- ✓ Basil
- ✓ Pomegranate
- ✓ Celery
- ✓ Vitamin B
- ✓ Horsetail tea
- ✓ Lemon juice, olive oil, raw apple cider vinegar
- ✓ Coconut water and barley water[44]

Herbal medicines act by multiple mechanisms like

- ❖ Helping in spontaneous passage of calculi by increasing urine volume, pH and anticalcifying activity (Diuretic activity).
- ❖ Balancing the Inhibitor and promoter of the crystallization in urine and affecting the crystal nucleation, aggregation and growth (Crystallization inhibition activity).
- ❖ Relieving the binding mucin of calculi (Lithotroptic activity).
- ❖ Improving renal function.
- ❖ Regulation of oxalate metabolism.
- ❖ Regulating the crystalloid-colloid imbalance and improving renal function, thus preventing recurrence of urinary calculi.
- ❖ Improving renal tissue antioxidant status and cell membrane integrity and preventing recurrence (Antioxidant activity).
- ❖ Exerting significant anti-infective action against the major causative organisms (Antimicrobial activity).
- ❖ ACE and Phospholipase A_2inhibition.
- ❖ Relieving symptoms like pain, burning micturition and haematuria[45] (Analgesic and anti-inflammatory activity).

Medicinal Herbs in the Treatment of Urolithiasis:

- *Ammi visnaga*
- *Pergularia daemia*
- *Momordica charantia*
- *Achyranthus aspera*
- *Moringa oliefera*
- *Quercus salicina*
- *Phyllanthus niruri*
- *Raphanus sativus*[44]

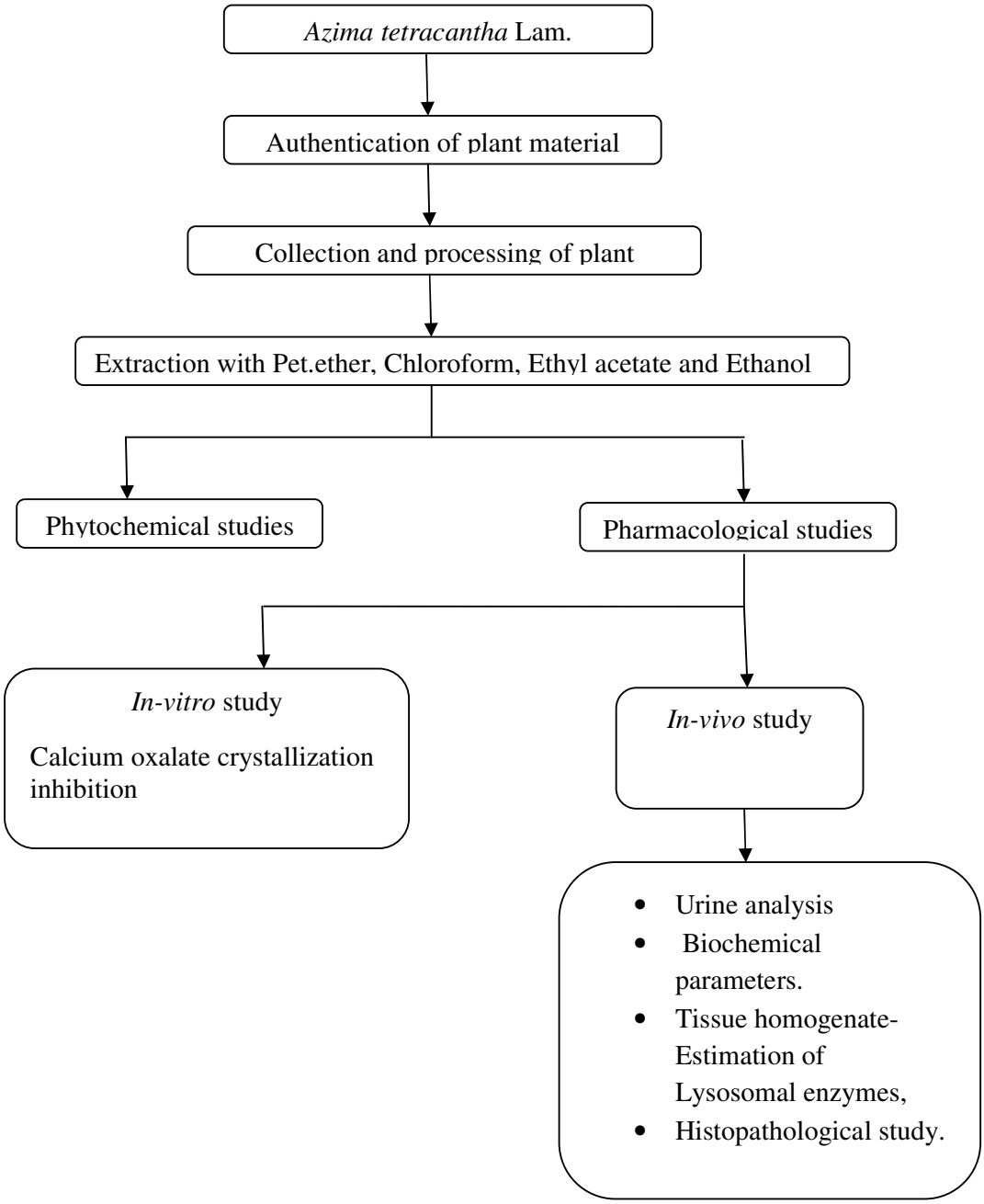
Available Herbal Formulations for Urolithiasis:

- ✓ Cystone
- ✓ Calcury
- ✓ Chandraprabha bati
- ✓ Trinapanchamool
- ✓ Rencare Capsule
- ✓ Patherina tablet
- ✓ Ber Patthar Bhasma
- ✓ Chander Prabha vati

It was felt that herbal medication to treat urolithiasis is the need of the hour. A standardised polyherbal formulation in a convenient dosage form along with scientific study would contribute significantly in the treatment of urolithiasis. In the present study, plant such as *Azima tetracantha* Lam. was selected for evaluate its antiurolithiatic activity.

4 MATERIALS AND METHODS

STUDY DESIGN



PLANT COLLECTION AND IDENTIFICATION

Dried whole plant of *Azima tetracantha. Lam* was collected from the forest around Panakkudi, Trinelveli District, Tamilnadu (INDIA), in the month of July 2013. It was authenticated by Prof. V. Chelladurai, Research Officer-Botany (Scientist-C) (RTD), Central Council for Research in Ayurveda and Siddha, Govt. of India.

PREPARATION OF PLANT EXTRACT:

The powdered plant material (50g) was extracted by Hot continuous soxhlet extraction method. The plant material was extracted with Ethanol (99.9% v/v) (500ml), Ethyl acetate (500ml), Chloroform (500ml) and Petroleum ether (500ml) for four days in a soxhlet apparatus[28].

It is a process of continuous extraction method in which the solvent can be circulated through the extractor for several times. The vapour from the solvent are taken to the condenser and the condensed liquid is returned to the extract for continuous extraction .The apparatus consist of body of extractor (thimble) attached with side siphon tube, lower end attached with distillation flask and the mouth of the extractor is fixed to the condenser by the standard joints.

Procedure:

- Weighed about 50g dried powdered plant and transferred into a thimble for packing.
- While packing, the content was wetted with Ethanol, Ethyl acetate, Chloroform and Petrolium ether respectively and poured until the siphon tube was filled.

- A piece of porcelain was added into the round bottom flask to avoid bumping effect.
- After assembling the extractor, the plant material was extracted at about (40-45°C), (35-40°C), (40-45°C), (20-30°C), temperature respectively until the colour of the solution in the siphon tube became pale.
- The extracts obtained were dried at room temperature and the yield was stored in air tight container.
- The Theoretical yield obtained was

Ethanol extract yield : 7.5 %w/w

Ethyl acetate extract yield : 5.2 %w/w

Chloroform extract yield : 6.3 %w/w

Petrolium ether extract yield: 2.8 %w/w

DRUGS AND CHEMICALS

Ethanol, Ethyl acetate, Chloroform, Petrolium ether, Cystone, Ethylene glycol.

4.1PHYTOCHEMICAL ANALYSIS[28]

1**. TEST FOR ALKALOIDS**:

A small portion of the extract was stirred with few drops of dilute hydrochloric acid and filtered. The filtrate was tested carefully with various alkaloidal reagents such as ***Mayer's reagent*** (cream precipitate), ***Dragendroff's reagent*** (orange brown precipitate), ***Hager's reagent*** **(**yellow precipitate), and ***Wagner's reagent*** (reddish brown precipitate).

2. TEST FOR FLAVONOIDS:

Schinado's test: To the extract, a few magnesium turnings and few drops of concentrated hydrochloric acid were added, boiled for 5minutes and red colour indicates the presence of flavonoids.

***Sodium hydroxide test*:** To the extract in ethanol, 10% sodium hydroxide was added. Dark yellow colouration indicates the presence of flavonoids.

3. TEST FOR TANNINS AND PHENOLIC COMPOUNDS

The extract was dissolved in distilled water. The extract was then divided into three parts. A sodium chloride solution 10% was added to one portion of test's extract, 1% gelatin solution to second portion and the gelatin salt reagent to third portion. Precipitation with the later reagent or with both the gelatin salt reagent was indicative of the presence of tannins. Precipitation of sodium chloride solution indicated a false-positive test. Positive tests were confirmed by the addition of a few drops of dilute ferric chloride (1% Ferric chloride) to test extracts, which gave black or green coloration.

The extract was mixed with lead acetate solution and observed for white precipitate.

4. TEST FOR TERPENOIDS

***Noller's test*:** The extract was warmed with tin and thionyl chloride. Pink coloration indicates the presence of terpenoids

5. TEST FOR GLYCOSIDES

***Borntrager's test*:** A small amount of extract was hydrolysed with hydrochloric acid for few hours on a waterbath and the hydrolysate was extracted with benzene. The benzene layer was treated with dilute solution and was treated with dilute solution and was observed for formation of reddish pink color.

***Legal's test*:** The extract was dissolved in pyridine and was made alkaline with few drops of 10% sodium hydroxide and then freshly prepared sodium nitroprusside was added and observed for the formation of blue color.

6. TEST FOR SAPONINS

Foam test: A small amount of extract was extracted with petroleum ether. To the insoluble residue left after extraction, a few ml of water was added and shaken vigorously for 15 minutes and was observed for the formation of honeycomb froth that persisted for at least 30 minutes.

7. TEST FOR PROTEIN:

Biuret test: The extract is treated with an equal volume of 1% strong sodium hydroxide followed by a few drops of copper (II) sulphate, formation of purple colour indicates the presence of protein.

Million's test: To the extract million's reagent is added, a white precipitate is produced, while heating it turns brick red colour, indicates the presence of protein.

8. TEST FOR MUCILAGE:

The extract is treated with aqueous potassium hydroxide, swelling indicates the presence of mucilage.

9. TEST FOR CARBOHYDRATES:

Molish's test: To the extract few drops of α-naphthol solution in alcohol, con.H_2SO_4 is added at the side of test tube, formation of violet ring at the junction of two liquids indicates the presence of carbohydrates.

10. TEST FOR PHYTOSTEROLS:

- ***Libermann – Burchard test:*** 1 mg of the extract was dissolved in few drops of chloroform, 3 ml of acetic anhydride and 3 ml of glacial acetic acid. Warmed, cooled under tap water and drops of concentrated sulphuric acid was added along the side of the test tube, formation of bluish green colour indicates the presence of steroids.

I) IN VITRO CALCIUM OXALATE CRYSTALLIZATION INHIBITION:[42,43]

Sample preparation: The various extraction of plant material was ready to use.

Experiment:

The precipitation of calcium oxalate at 37^{o}C and pH 6.5 has been studied by the measurement of turbidity at 620nm. A spectrophotometer UV/Vis (SHIMADZU 1800) was employed to measure the developed turbidity due to the formation of calcium oxalate.

Chemicals used:

Chemicals used were of pure and analytical grade.

1. Calcium chloride dehydrate ($CaCl_2.2H_2O$)
2. Sodium oxalate ($Na_2C_2O_4$)
3. Sodium chloride (NaCl)

Procedure:

Study without inhibitor:

The solutions of $CaCl_2.2H_2O$ (10mM) and $Na_2C_2O_4$ (4mM) were prepared using sodium chloride solution (0.15M). A volume of 1.5ml of calcium chloride dehydrate was transferred in to the cell and blank reading was taken. 1.5ml of sodium oxalate solution was added to the previous volume, and the turbidity measurement was immediately started for a period of 10min. For each experiment, six replicates were taken.

Study with inhibitor:

The inhibitor (100%) was prepared by taking 0.5g of the extract with 60ml of sodium chloride (0.15mM). From this inhibitor, prepare diluted inhibitory solutions (10%, 50%)

using solvent such as sodium chloride solution (0.15mM). A mixture of 1ml of calcium chloride dehydrate (10mm) and 1ml of inhibiting solution was versed in the cell. Take a blank reading, and then a volume of 1ml of sodium oxalate (4mM) was added and the measurement was immediately started for a period of 10minutes.

The % of inhibition was calculated using the following formula:

$$\mathbf{I\ (\%) = [1-Ti/\ Tc]\ x\ 100}$$

Where,

Ti is turbidimetric slope with inhibitor,

Tc is turbidimetric slope without inhibitor.

Microscopic study:

The photographs were taken using a microscope optic equipped with a digital camera and connected with a microcomputer. At a time corresponding to the stage of growth and aggregation, a drop of the mixture of crystallizable solution, or inhibitory solution was placed in the glass slide, which was immediately placed under the objective of the microscope.

II) *IN-VIVO* ANTIUROLITHIASIS ACTIVITY OF VARIOUS EXTRACT OF *Azima tetracantha* Lam. ON ETHYLENE GLYCOL INDUCED UROLITHIASIS IN RATS[46,47,48,49]

Experimental Animals:

The present study was conducted after obtaining approval from the Institutional Animal Ethics Committee and this protocol met the requirements of national guidelines of CPCSEA (PROPOSAL NO: Vide. 14/243/CPCSEA). The Wistar albino rats (150-200g) used for this study were procured from Animal house, Madras Medical College, Chennai, India.

Quarantine and Acclimatization:

Quarantine is the separation of newly received animals from those already in the facility until the health and possibly the microbial status of the newly received animals have been determined. The newly procure Wistar albino rats were quarantined for the period of one week to minimize the chance of introduction of pathogens into established animals and allowed to develop the psychological, physiological and nutritional stabilization before their use.

Housing:

The animals were housed in well ventilated animal house which was maintained at a constant temperature and relative humidity of 55 to 60%. The animals were housed in spacious polypropylene cages and paddy husk was utilized as bedding material.

Diet and water:

The animals were maintained on standard pellet diet and purified water. The animals were provided with food and water ad libitum except during fasting. The bed material was changed twice a week.

Animal identification:

All animal cages used in the study had a proper identification i.e., labels. Each animal in the cage was marked either on head or body or tail with picric acid for their appropriate identification.

***IN-VIVO* ANTIUROLITHIASIS EVALUATION**

The antiurolithiasis activity of *Azima tetracantha* Lam. was evaluated in urolithiatic wistar rats. Urolithiasis was induced by oral administration of ethylene glycol (0.75% v/v) in

drinking water. The antiurolithiatic effect of the plant extract was compared with standard drug Cystone.

Ethylene Glycol Induced Urolithiasis Model[46,47,48,49]

After a week of acclimatization, the rats were divided into seven groups containing six animals in each.

Table 4.1 In vivo anti-urolithiasis Experimental design

Group	Treatment	No.of animals
I	Normal control	6
II	Ethylene Glycol (0.75% v/v) for 28 days	6
III	Ethylene Glycol (0.75% v/v) for 28 days + standard drug Cystone 750mg/kg bw,p.o (15-28th day)	6
IV	Ethylene Glycol (0.75% v/v) for 28 days + Ethanolic extract of AT 200mg/kg bw,p.o (15-28th day)	6
V	Ethylene Glycol (0.75% v/v) for 28 days + Ethyl acetate extract of AT 200mg/kg, bw,p.o (15-28th day)	6
VI	Ethylene Glycol (0.75% v/v) for 28 days + Chloroform extract of AT 200mg/kg bw,p.o (15-28th day)	6
VII	Ethylene Glycol (0.75% v/v) for 28 days + Petrolium extract of AT 200mg/kg bw,p.o (15-28th day)	6

Assessment of Antiurolithiatic Activity

On 28thday all animals which were kept in metabolic cages are taken and urine samples were collected. Animals had free access to drinking water during the urine collection

period. A drop of concentrated hydrochloric acid was added to the urine before being stored at 4°C. Urine was analyzed for urine volume, pH, calcium, phosphate, oxalate and magnesium content using the method of Bahuguna *et al.,*[50].

4.3.1 URINE VOLUME

Animals were placed in separate metabolic cages for 24h and total urinary volume was measured using the cylinder and reported in ml[50].

4.3.2 URINE pH

Uric acid crystals were found to deposit most frequently in the concentrated acid urine. Thus, the acidity of the urine was tested using pH meter[50].

ESTIMATION OF CALCIUM IN URINE (BioChain)

CALCIUM ASSAY KIT (Z5030014)

Calcium is measured to moniter diseases of the bone or calcium regulation disorders. Increased calcium levels in serum are reported in hyperparathyroidism, metabolic bone lesions and hypervitaminosis, while decreased levels are observed in hypoparathyroidism, nephrosis, rickets, steatorrhea, nephritis and calcium-losing syndromes.

Urinary calcium levels aid the clinician in understanding how the kidneys handle calcium in certain diseases of the parathyroid gland. Urinary calcium levels are also essential in the medical evaluation of kidney stones.

Principle

Simple, direct and automation-ready procedures for measuring calcium concentration in biological samples are becoming popular in Research and Drug Discovery. Biochain's calcium assay kit is designed to measure calcium directly in biological samples without any pretreatment. A phenosulphonephthalein dye in the kit forms a very stable blue colored complex specifically with free calcium. The intensity of the color, measured at 612nm is

directly proportional to the calcium concentration in the sample. The optimized formulation minimizes any interference by substances such as magnesium, lipid, protein and bilirubin.

Kit contents (500 tests in 96-well plates)

Reagent A: 50ml

Reagent B: 50ml

Calcium standard: 1mL 20mg/dl Ca^{2+}

Storage conditions:

The kit is shipped at room temperature. Store reagent and standard at 4^{o}c. Shelf life: 12 months after receipt.

Procedure

Reagent Preparation:

Prepare enough working reagent by combining equal volumes of reagent A and B. Equilibrate to room temperature before use.

Procedure using 96-well plate:

1. Dilution:

   Transfer 5ml diluted standards and samples into wells of a clear bottom 96-well plate. Store diluted standards at 4^{o}c for future use.

Premix	H_2O	Ca (mg/dl)
100ml + 0ml	100	20
80ml + 20ml	100	16
60ml + 40ml	100	12
40ml + 60ml	100	8
30ml + 70ml	100	6
20ml + 80ml	100	4
10ml + 90ml	100	2
0ml + 100ml	100	0

2. Add 200ml working reagent and tap lightly to mix.

3. Incubate 3min at room temperature and read optical density at 570- 650nm (peak absorbance at 612nm).

Procedure using cuvette:

1. Set up tubes for diluted standards and samples. Transfer 15ml diluted standards and samples to appropriately labeled tubes.

2. Add 1000ml working reagent and vortex to mix. Incubate 3min. Transfer to cuvet and read optical density at 612nm.

CALCULATION

Substrate blank OD from the standard OD values and plot the OD against Ca^{2+} standard concentrations. Determine the slope using linear regression fitting. Calcium concentration of the sample is calculated as

$$\mathbf{=OD_{sample} - OD_{blank}/Slope.}$$

OD_{sample} and OD_{blank} are read at OD 612nm values of sample and blank (water or buffer in which the sample was diluted).

Conversions: 1mg/dl Ca^{2+} equals 250mM, 0.001% or 10ppm.

ESTIMATION OF MAGNESIUM IN URINE

MAGNESIUM KIT (Calmagite method)

Magnesium, along with potassium, is a major intracellular cation. It is an activator of various enzymes. It is also involved in amino acid activation and protein synthesis. Increased levels are found in dehydration, addition's disease and uremia. Decreased levels are found in

malabsorption, during treatment of diabetic coma, chronic renal disease, chronic alcoholism, pancreatitis and hyperthyroidism.

Principle:

Magnesium combines with Calmagite in an alkaline medium to form a red colored complex. Interference of calcium and proteins are eliminated by the addition of specific chelating agents and detergents. Intensity of the colour formed is directly proportional to the amount of magnesium present in the sample.

Magnesium + Calmagite —(**alkaline Medium**)→ **Red colour complex**

Contents:	**25ml**	**75ml**
L1: Buffer reagent	12.5ml	37.5ml
L2: Colour reagent	12.5ml	37.5ml
S: Magnesium standard (2.0 mEq/L)	2ml	2ml

Storage/ stability:

Contents are stable at 2-8^{o}c till the expiry mentioned on the label.

Reagent preparation:

Reagents are ready to use. Protect from bright light.

Working reagent: For large assay series a working reagent may be prepared by mixing equal volume of L_1 (Buffer reagent) and L_2 (colour reagent). The working reagent is stable at 2-8^{o}c for at least one month keep tightly closed.

Sample material:

Urine: 24hr. collected urine should be acidified to a pH of 2-3 by the addition of approx. 10-15 ml of HCl and diluted 1+3 with distilled water before use. Multiply results by 4.

Procedure:

Wavelength / filter : 510nm

Temperature : Room temperature

Light path : 1cm

Pipette into clean dry test tubes labeled as Blank (B), Standard (S) and Test(T).

Addition sequence	Blank (ml)	Standard (ml)	Test (ml)
Buffer reagent (L1)	0.5	0.5	0.5
Colour reagent (L2)	0.5	0.5	0.05
Distilled water	0.01	-	-
Magnesium standard (S)	-	0.01	-
Sample	-	-	0.01

Mix well and incubate at room temperature (25^{o}c) for 5min. Measure the absorbance of Standard (Abs.S) and Test (Abs.T) against the blank, within 30min.

System parameters:

Reaction : End point Sample volume: 0.01ml

Wave length : 510nm Reagent volume: 1.00ml

Zero setting : Reagent Blank Reaction slope : Increasing

Incubator. Temp : Room temp Linearity : 10mEq/ L

Incubation time : 5min Units : mEq/ L

Standard : 2.0mEq/L

Calculation:

$$\text{Magnesium in mEq/L} = \frac{\text{Abs. T}}{\text{Abs. S}} \times 2$$

Levels of calcium, magnesium in urine and urea, uric acid, creatinine in serum were estimated by standard kits of **CREST BIOSYSTEMS**, Goa, India.

ESTIMATION OF PHOSPHORUS IN URINE

PHOSPHORUS KIT (Molybdate U.V method)

Phosphorus is mainly combined with calcium and is found in the bones. Approximately 15% exists as inorganic phosphorus or phosphate esters. It is involved in the carbohydrate metabolisms and is a component of many other substances. Increased levels are found in hypothyroidism, renal failure, bone metastasis and liver disease. Decreased levels are found in hyperthyroidism, rickets and vitamin D deficiency.

Principle:

Phosphate ions in an acidic medium react with ammonium molybdate to form a phosphomolybdate complex. This complex has an absorbance in the ultraviolet range and is measured at 340nm. Intensity of the complex is directly proportional to the amount of inorganic phosphorus present in the sample.

Phosphorus + Ammonium Molybdate ----------→ Phosphomolybdate complex

Contents	25ml	2 x 75ml
L1: Acid Reagent	60ml	2 x 60ml
L2: Molybdate Reagent	15ml	2 x 15ml
S: Phosphorus Standard (5mg/dl) 2ml	5ml	5ml

Storage/ stability:

Contents are stable at room temperature (25-30^{o}c) till the expiry mentioned on the label.

Reagent preparation

Reagents are ready to use.

Working Reagent:

Pour the contents of 1 bottle of L_2 (Molybdate Reagent) into 1 bottle of L_1 (Acid Reagent). This working reagent is stable for at least 6 months when stored at 2-8^{o}C. Upon storage this working reagent may develop a slight blue colour however this does not affect the performance of the reagent.

Alternatively for flexibility as much of working reagent may be made as and when desired by mixing together 4 parts of L_1 (Acid reagent) and 1 part of L_2 (Molybdate reagent). Alternatively 0.8ml of L_1 and 0.2ml of L_2 may also be used instead of 1ml of the working reagent directly during the assay.

Sample material:

Urine: Acidify the urine with a few drops of conc. HCl and dilute 1+ 19 before the assay (results x 20).

Procedure:

Wavelength/ filter : 340nm

Temperature : Room temperature

Light path : 1cm

Pipette into clean dry test tubes labeled as Blank (B), Standard (S) and Test (T).

Addition sequence	Blank (ml)	Standard (ml)	Test (ml)
Working reagent	1.0	1.0	1.0
Distilled water	0.01	-	-
Phosphorus Standard (S)	-	0.01	-
Sample	-	-	0.01

Mix well and incubate at room temperature for 5min. Measure the absorbance of the standard (Abs. S) and Test Sample (Abs. T) against the blank, within 60min.

System parameters:

Reaction : UV End point Sample volume: 0.01ml

Wave length : 340nm Reagent volume: 1.00ml

Zero setting : Reagent Blank Reaction slope : Increasing

Incubator. Temp : Room temp Linearity : 20mg/dl

Incubation time : 5min Unit : mg/dl

Standard : 5mg/ dl

Calculation:

$$\text{Magnesium in mg/ dl} = \frac{\text{Abs. T}}{\text{Abs. S}} \times 5$$

4.4 SERUM ANALYSIS

After the experimental period, blood was collected from the retro-orbital under anesthetic conditions. The blood was collected and serum was separated by centrifugation at 10,000 rpm for 10min. The serum supernatant was collected and then diluted within the ratio of 1:10. Aliquots of the diluted serum were used for the determination of serum constituents like creatinine, uric acid and urea nitrogen using the method of Atef and Attar and serum enzyme activities[51].

4.5 COLLECTION OF KIDNEY SAMPLES

At the 29th day, one animal from each experimental animal groups were sacrificed, kidney were removed immediately and washed with ice cold saline. 10% tissue homogenate was prepared by homogenizing 1g of chopped kidney tissue in 10ml of 0.1M tris HCl homogenizing buffer at pH 7.5. The homogenate was used for assaying the enzyme activities[(52)].

Chemicals:

All the chemicals used in the present study were of analytical reagent grade.

4.6 ESTIMATION OF BIOCHEMICAL MARKERS[(53,54,55)]

The serum and tissue homogenate was used to assay the marker enzymes in serum and tissue constituents like ACP, ALP, AST, ALT and LDH according to the method of King

et al., (1965a), King and Armstrong (1934), Reitman and Frankel (1957), Reitman and Frankel (1957) and King (1965b) respectively.

ESTIMATION OF UREA AND BLOOD UREA NITROGEN

UREA KIT

Urea is the end product of the protein metabolism. It is synthesized in liver from the ammonia produced by the catabolism of ammonia acids. It is transported by the blood to the kidneys from where it is excreted. Increased levels are bound in renal diseases, urinary obstructions, shock, congestive heart failure and burns. Decreased levels are found in liver failure and pregnancy.

Principle: Urea is an acidic medium condenses with Diacetyl monoxime at 100^{o}c to form a red coloured complex. Intensity of the colour formed is directly proportional to the amount of urea present in the sample.

Urea + Diacetyl monoxime ----------→ Red Coloured Complex

Contents:	**25ml**	**75ml**
L_1: Urea Reagent	75ml	150ml
L_2: Acid Reagent	75ml	150ml
L3: DAM Reagent	75ml	150ml
S: Urea Standard (40mg/dl)	5ml	5ml

Storage/ stability:

All reagents are stable at room temperature till the expiry mentioned on the label.

Reagent Preparation:

Reagents are ready to use. Do not pipette with mouth.

Sample material:

Serum. Urea reported to be stable in the serum for 5 days when stored at 2-8^{o}c.

Procedure:

Wavelength/ filter : 520nm (Hg 546 nm) / Green

Temperature : 100^{o}c

Light path : 1cm

Addition sequence	Blank (B) ml	Standard (S) ml	Test (T) ml
Urea reagent (L_1)	1.0	1.0	1.0
Acid reagent (L_2)	1.0	1.0	1.0
DAM reagent	1.0	1.0	1.0
Distilled water	0.01	-	-
Urea Standard (S)	-	0.01	-
Sample	-	-	0.01

Pipette into clean dry test tubes labeled as Blank (B), Standard (S) and Test (T).

Mix well and keep the test tubes in boiling water (100^{o}c) for 10min. Cool under running tap water and measure the absorbance of the standard (Abs. S) and Test sample (Abs. T) against the blank.

System parameters:

Reaction : End point Sample volume: 0.01ml

Wave length	: 520nm	Reagent volume: 3.00ml	
Zero setting	: Reagent Blank	Reaction slope	: Increasing
Incubator. Temp	: 100^{o}c	Linearity	: 70mg/dl
Incubation time	: 10min	Unit	: mg/dl
Standard	: 40mg/ dl		

Calculation:

$$\textbf{Urea in mg/ dl} = \frac{\textbf{Abs. T}}{\textbf{Abs. S}} \textbf{ X 40}$$

Blood Urea Nitrogen = Urea in mg/ dl x 0.467

ESTIMATION OF URIC ACID IN SERUM

URIC ACID KIT (Uricase / PAP method)

Uric acid is the end product of purine metabolism. Uric acid is excreted to a large degree by the kidney and to a smaller extent in the intestinal tract by microbial degradation. Increased levels are found in Gout, arthritis, impaired renal functions and starvation. Decreased levels are found in Wilson's disease, Fanconis syndrome and yellow atrophy of liver.

Principle:

Uricase converts uric acid to allantoin and hydrogen peroxide. The hydrogen peroxide formed further reacts with a phenolic compound and 4 aminoantipyrine by the catalytic action of peroxidase to form a red coloured quinoneimine eye complex. Intensity of the colour formed is directly proportional to the amount of uric acid present in the sample.

Uricase

Uric acid + H_2O ----------→ Allantoin + H_2O_2

Peroxidase

H_2O_2 + 4 Aminoantipyrine ----------→ Red Quinoneimine dye + H_2O

+ Phenolic compound

Contents:	**25ml**	**75ml**	**2X75ml**	**2X150ml**
L1: Buffer reagent	20ml	60ml	2X60ml	2X120ml
L2: Enzyme reagent	5ml	15ml	2X15ml	2X30ml
S: Uric acid Standard (8mg/ dl)	5ml	5ml	5ml	5ml

Storage/ stability:

All reagents are stable at 2-8^{o}c till the expiry mentioned on the label.

Reagent Preparation:

Reagents are ready to use.

Working reagent:

Pour the contents of 1 bottle of L_2 (Enzyme Reagent) into 1 bottle of L_1 (Buffer reagent). This working reagent is stable for at least 4 weeks when stored at 2-8^{o}c. Upon storage this working reagent may develop a slight pink colour however this does not affect the performance of the reagent.

Alternatively for flexibility as much of working reagent may be made as and when desired by mixing together 4 parts of L_1 (Buffer reagent) and 1 part of L_2 (Enzyme reagent).

Alternatively 0.8ml of L_1 and 0.2ml of L_2 may also be used instead of 1ml of the working reagent directly during the assay.

Sample material:

Serum. Uric acid is reported to be stable in the sample for 3-5 days when stored at 2-8^{o}c.

Procedure:

Wave length/ filter : 520nm / Yellow Green

Temperature : 37^{o}c / Room temperature

Light path : 1cm

Addition sequence	Blank (ml)	Standard (ml)	Test (ml)
Working reagent	1.0	1.0	1.0
Distilled water	0.02	-	-
Phosphorus Standard (S)	-	0.02	-
Sample	-	-	0.02

Pipette into clean dry test tubes labeled as Blank (B), Standard (S) and Test (T).

Mix well and keep the test tubes in boiling water (100^{o}c) for 10min. Cool under running tap water and measure the absorbance of the standard (Abs. S) and Test sample (Abs. T) against the blank.

System parameters:

Reaction : End point Sample volume: 0.02ml

Wave length : 520nm Reagent volume: 1.00ml

Zero setting	: Reagent Blank	Reaction slope	: Increasing
Incubator. Temp	: 37^{o}c/ room temp.	Linearity	: 20mg/dl
Incubation time	: 5 min / 15 min	Unit	: mg/dl
Standard	: 8mg/ dl		

Calculation:

$$\text{Urea in mg/ dl} = \frac{\text{Abs. T}}{\text{Abs. S}} \times 8$$

ESTIMATION OF CREATININE IN SERUM

CREATININE KIT (Alkaline Picrate method)

Creatinine is the catabolic product of creatinine phosphate which is used by the skeletal muscle. The daily production depends on muscular mass and it is excreted out by the body entirely by the kidneys. Elevated levels are found in renal dysfunction, reduced renal blood flow (shock, dehydration, congestive heart failure), diabetes acromegaly. Decreased levels are found in muscular dystrophy.

Principle:

Picric acid in an alkaline medium reacts with creatinine to form a coloured complex with the alkaline picrate. Intensity of the colour formed is directly proportional to the amount of creatinine present in the sample.

Creatinin + Alkaline picrate ----------→ Orange Red Coloured Complex

Contents:	**15 tests**	**35 tests**	**70 tests**
L_1: Picric acid reagent	60ml	140ml	2 x140ml
L_2: Buffer reagent 75ml	5ml	12ml	25ml
S: Creatinine standard (40mg/dl)	5ml	5ml	10ml

Storage/ stability:

All reagents are stable at room temperature till the expiry mentioned on the label.

Reagent preparation:

Reagents are ready to use. Do not pipette with mouth.

Sample material:

Serum. Creatinine is stable in serum for 1 day at 2-8^{o}c.

Procedure:

Wavelength/ filter : 520nm / Green

Temperature : Room temperature

Light path : 1cm

Deproteinization of specimen:

Pipette into a clean dry test tube

Picric acid reagent (L_1)	2.0ml
Sample	0.2ml

Pipette into clean dry test tubes labeled as Blank (B), Standard (S) and Test (T).

Addition sequence	Blank (B) ml	Standard (S) ml	Test (T) ml
Supernatant	-	-	1.0
Picric acid reagent (L_1)	1.0	1.0	-
Distilled water	0.1	-	-
Creatinine standard (S)	-	0.1	-
Sample	-	-	0.1

Mix well and keep the test tubes at room temperature for exactly 20min. Measure the absorbance of the standard (Abs. S) and Test sample (Abs. T) against the blank.

System parameters:

Reaction : End point Sample volume: 0.1ml

Wave length : 520nm Reagent volume: 1.1ml

Zero setting : Reagent Blank Reaction slope : Increasing

Incubator. Temp : Room temp Linearity : 8mg/ dl

Incubation time : 20min Units : mg/ dl

Standard : 8mg/ dl

Calculation:

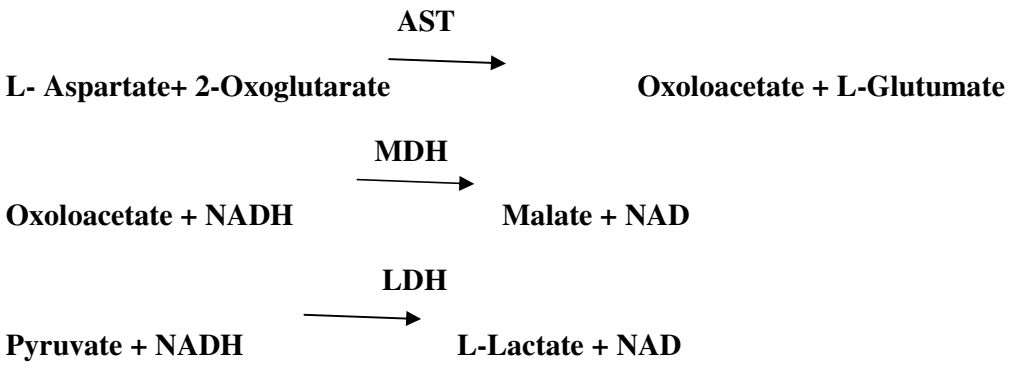
Creatinine in mg % = Abs. T / Abs. S x 2

ESTIMATION OF SGOT IN SERUM[54]

IFCC METHOD, KINETIC

The aminotransferases (transaminases) are widely distributed in animal tissues. Both AST and ALT are normally present in human plasma, bile, cerebrospinal fluid and saliva. Elevated AST levels are observed in viral hepatitis and other liver disease, cirrhosis, myocardial infarction.

Principle:



AST: Aspartate aminotransferase

MDH: Malate dehydrogenase

LDH: Lactate dehydrogenase

KIT COMPONENTS

1. R_1- SGOT Reagent 1

2. R_2- SGOT Reagent 2

Composition in the test: Tris buffer 80mmol/ l pH 7.8

L- aspartate 200mmol/ l

2- Oxoglutarate 12mmol/ l

NADH 0.18mmol/ l

MDH > 600 U/ l

LDH > 900 U/ l

Stabilizers.

Reagent preparation, storage and stability:

Mix reagent 1& reagent 2 in ratio 4:1. Keep away from direct light sources.

Stability: up to expiration date on label at 2-8^{o}c.

Stability of working reagent: 30days at 2-8^{o}c.

Reagent deterioration:

Discard the working reagent if absorbance < 0.8 at 340nm against distilled water.

Specimen:

Use serum, plasma. SGOT is stable for 4days at 2-8^{o}c or 1 month at -20^{o}c.

Test procedure:

Dispense in tube: working reagent	1000 micro litre
Add sample	50 micro litre
Mix and incubate 60 seconds at 37^{o}c, then record first reading of absorbance. Perform other readings at 60 seconds intervals. Calculate the ΔA/ min.	

System parameters

Reading mode	Rate	Volume	500 micro liter
Factor	3376	Delay time	60sec
Wave length	340nm	Read time	180sec
Temperature	37^{o}c	Unit	U/ l
Reaction direction	Decrease	Reference Low	0
Linearity Limit	1000	Reference High	45

Result calculation

Perform calculations in units per litre, multiplying the ΔA/ min by the factor.

Activity in U/ l = ΔA/ min x 3376

ESTIMATION OF ALKALINE PHOSPHATASE IN SERUM[53]

Modified IFCC, Kinetic:

Alkaline phosphatase occurs high levels in liver, bone, intestine and placenta. Increased levels occur in hepatobiliary diseases and bone diseases. Elevated ALP occurs in pregnant women and growing children.

Principle:

The enzyme Alkaline phosphatase hydrolizes the 4- Nitro phenol phosphate to release 4-nitrophenol, under alkaline conditions. The 4- Nitro phenol formed is detected spectrophotometrically at 405 nm to give a measurement of alkaline phosphatase activity in the sample.

2- amino-2-methyl-1-propanol + p- nitrophenylphosphate + H_2O

4 –nitrophenol + 2-amino-2-methyl-1-propanol phosphate

Kit components

Composition:

Alkaline phosphatase reagent: 2- amino-2-methyl-1-propanol buffer 0.35 mol/ l –ph 10.40

Magnesium acetate 2mmol/ l

Zinc sulfate 1mmol/ l

HEDTA 2mmol/ l

4- NPP 16mmol/ l

Reagent preparation, storage and stability:

Reagent is ready to use. Keep away from direct light sources.

Stability: up to expiration date on label at 2-8^{o}c.

Stability since first opening of bottle at 2-8^{o}c.

Test procedure:

Dispensing reagent in tube	1ml
Sample	0.02ml

Mix and execute a first reading of absorbance after 1minute, incubating at 37^{o}c. Perform other 2 readings at 60seconds intervals. Calculate the ΔA/ min.

System parameters:

Reading mode	End point	Sample Volume	20 micro liter
Factor	2764	Wave length	405nm
Temperature	37^{o}c	Unit	U /l
Reagent volume	1ml	Reference Low	42
Linearity Limit	1200	Reference High	128

Calculation:

Alkaline phosphatase U/ l = ΔA/ min x 2764

LACTATE DEHYDROGENASE (LDH) ASSAY[52,62]

Principle

The assay was performed according to King's et al., (1965) method, when the enzyme is supplied with Pyruvate and $NADH^{+}$, the LDH catalyzed reaction starts to produce lactate. At certain time point the reaction is terminated by the addition of 2,4-dinitrophenylhydrazine, which reacts with lactate at acidic p^{H}. After alkalization (addition of NaOH) the resulting hydrazine derivate gives a yellowish-orange colour suitable for quantification by means of spectrophotometry at the wavelength of 440 nm.

Requirements

- 0.1M phosphate buffer (p^{H} 7.5)
- NADH(6.6 mM)
- Sodium pyruvate (30mM)
- Dinitro phenyl hydrazine

Procedure

0.1ml of tissue homogenate was added with 2.7ml buffer, 0.1ml NADH and 0.1ml sodium pyruvate. The mixture was heated for 15mins at 37°C. Then 0.5ml of dinitro phenyl hydrazine was added and incubated at room temperature for 15mins. The reaction was stopped by addition of 5ml 0.1N NaOH. The developed colour was measured at 440nm.

LYSOSOMAL ENZYMES ESTIMATION IN TISSUE PREPARATIONS

ASSAY OF ALKALINE PHOSPHATASE (ALP)[61]

Principle

When the enzyme incubated with p-nitro phenyl phosphate and Tris buffer (p^H 9.6), in alkaline condition inorganic phosphate and p-nitro phenol are formed by the catalytic action of alkaline phosphatase. Amount of p-nitro phenol liberated by the enzyme is measured at 420 nm.

2-amino-methyl-1-propanol + p-nitro phenyl phosphate + H_2O

4-nitro phenol + 2-amino-2-methyl-1-propanol phosphate

Requirements

- P-nitro phenyl phosphate (10 mM)
- Tris-HCl p^H 9.6 (80mM)
- NaOH (0.1N)

Procedure

1ml of p-nitro phenyl phosphate and 1.5ml of buffer were added with 100µl of homogenate. The mixture was incubated at 37°C for 30mins. Then the reaction was stopped

by addition of 0.1 N NaOH. The absorbance of liberated p-nitro phenol was measured at 420nm.

Calculation

ALP U/I = ΔA/ 2764

ASSAY OF ACID PHOSPHATASE (ACP)[60]

(α-Naphthylphosphate Kinetic method)

Principle

In acidic condition, the incubation of α-Naphthylphosphate with ACP will liberate α –Naphthol and inorganic phosphate due to catalytic action of ACP. The –Naphthol formed is coupled with Fast Red to form a diazo dye complex. The rate of formation of this complex is measured as an increase in absorbance which is proportional to the ACP activity in the sample.

α –Naphtholphosphate + H_2O $\xrightarrow{ACP}$ α –Naphthol + Phosphate

α –Naphthol + Fast Red TR Sal ⟶ Diazo dye complex

Requirements

- α –Naphthol phosphate (4.5mM)
- acetate buffer (p^H 5.0)
- NaOH (0.2N)

Procedure

1ml of α –Naphthol and 1.9 ml of buffer were added with 100µl of homogenate. The mixture was incubated at 37°C for 30mins. The absorbance of liberated α –Naphthol was measured at 420 nm.

Calculation

ACP activity in U/L = ΔA/min x 750

ESTIMATION OF ASPARTATE AMINOTRANSFERASE (AST/ SGOT)[59]

Aspartate transaminase (AST) also referred to serum glutamate oxaloacetate transferase (SGOT) is an enzyme involved in amino acid metabolism. AST is widely distributed in liver, RBCs, heart, pancreas and kidney. A low level of SGOT in blood is observed in severe liver disease, myocardial infarction, heart failure, kidney disease and lung disease.

Principle

α-Ketoglutarate + L-Aspartate $\xrightarrow{\text{SGOT}}$ **L-Glutamate + Oxaloacetate**

Oxaloacetate + NADH+ H^+ $\xrightarrow{\text{MDH}}$ **L-Malate + NAD^+**

The rate of NADH consumption is measured photometrically and is directly proportional to the SGOT concentration in the sample.

Reagents

- L-Aspartate >200mmol/l
- Malate dehydrogenase > 200mmol/l
- α-Ketoglutarate>35mmol/l
- NADH >1.05 mmol/l

Procedure

800µl of L-Aspartate & Malate dehydrogenase and 200µl of α-Ketoglutarate are mixed together and incubated at 37°C for 2minutes and 100µl of sample is added. The change in absorbance is measured at 340nm.

Calculation

AST = Δ Abs/ min x Factor (1746)

ESTIMATION OF ALANINE AMINOTRANSFERASE (ALT/ SGPT)[58]

Alanine aminotransferase / serum glutamate pyruvate transferase ia an enzyme involved in amino acid metabolism.

Principle

α-Ketoglutarate + L-Alanine $\xrightarrow{\text{SGPT}}$ **L-Glutamate + Pyruvate**

Pyruvate + NADH+ H^+ $\xrightarrow{\text{LDH}}$ **L-Lactate + NAD^+**

The rate of NADH consumption is measured photo metrically and is directly proportional to the SGPT concentration in the sample.

Reagents

- L-Alanine >200mmol/l
- Lactate dehydrogenase > 1500mmol/l
- α-Ketoglutarate>35mmol/l
- NADH >1.05 mmol/l
- Tris buffer 80mmol/l pH 7.5

Procedure

800µl of L-Alanine & Lactate dehydrogenase and 200µl of α-Ketoglutarate are mixed together and incubated at 37°C for 2minutes and 100µl of sample is added. The change in absorbance is measured at 340nm.

Calculation

ALT = Δ Abs / min x Factor (1746)

III) HISTOPATHOLOGICAL ASSAY[31,63]

At the 29th day, one rat from each group was sacrificed and both kidneys of each rat were removed for histopathologic examination. Removed kidneys were fixed in 10 % buffered formalin (MERK) over night and then each was sliced longitudinally in 3 sections, including anterior, middle and posterior parts of the kidney. Thereafter, they were

automatically processed and inserted in paraffin blocks and at least 6 sections with 5 micron thickness were obtained from each kidney and stained by Hematoxylin and Eosin.

IV) Statistical analysis[31]

Results were expressed as mean ± S.D. Differences among data were determined using one-way ANOVA followed by Dunnet's test. Differences between the data were considered significant at $p< 0.05$.

5. RESULTS AND DISCUSSION

PRELIMINARY PHYTOCHEMICAL ANALYSIS

TEST	Ethanol Ext.	Ethyl acetate Ext.	Chloroform Ext.	Pet.ether Ext.
1.TEST FOR FLAVONOIDS a) Shinado's test b) Sodium hydroxide test	+	+	+	+
2.TEST FOR TANNINS With lead acetate	+	+	-	-
3.TEST FOR SAPONINS Foam test	+	+	-	-
4.TEST FOR TERPENOIDS With Tin and thionyl chloride	+	+	-	-
5.TEST FOR GLYCOSIDES a) Libermann-burchard's test b) Legal's test c) Borntrager's test	-	-	-	-
6.TEST FOR PHYTOSTEROLS Libermann test	+	+	+	+
7.TEST FOR MUCILAGE Swelling test	-	-	-	-
8.TEST FOR PROTEIN a) Biuret test b) Million's test	+	+	+	+
9.TEST FOR CARBOHYDRATES Molish's test	+	+	+	-

10.TEST FOR ALKALOIDS a) Drangendroff's test b) Mayer's test c) Hager's test d) Wagner's test	+	+	+	-

(+present) **(-absent)**

Table 5.1Preliminary phytochemical analysis of various extract of *Azima tetracantha* Lam.

5.1 *INVITRO* ANTIUROLITHIATIC ACTIVITY:

CALCIUM OXALATE CRYSTALLIZATION INHIBITION BY VARIOUS EXTRACT OF WHOLE PLANT OF *Azima tetracantha* Lam.

The effect of various extract of plant extract on various phases of calcium oxalate crystallization was determined by time course measurement of turbidity in the sodium chloride solution. The absorbance according to the time for tries without and with inhibitor was represented in the graph taking time vs. absorbance. The values of the change in absorbance for all the extract were noted and a graph was plotted.

Table 5.2 Calcium oxalate crystallization inhibition with the formulation

S.No	Time in Secs	Without Inhibitor	Ethanol	Ethyl acetate	Chloroform	Pet.ether
1	30	0.16	0.04	0.09	0.10	0.14
2	60	0.22	0.17	0.25	0.38	0.40
3	90	0.45	0.19	0.35	0.40	0.42
4	120	0.72	0.21	0.39	0.50	0.65
5	150	0.82	0.25	0.45	0.58	0.72
6	180	0.93	0.29	0.56	0.66	0.76
7	210	0.97	0.33	0.60	0.68	0.80
8	240	0.99	0.35	0.62	0.68	0.82
9	270	0.99	0.35	0.62	0.70	0.82
10	300	0.99	0.35	0.62	0.70	0.82
11	330	0.99	0.35	0.62	0.70	0.82
12	360	0.99	0.35	0.62	0.70	0.82
13	390	0.99	0.35	0.62	0.70	0.82
14	420	0.99	0.35	0.62	0.70	0.82
15	450	0.99	0.35	0.62	0.70	0.82
16	480	0.99	0.35	0.62	0.70	0.82
17	510	0.99	0.35	0.62	0.70	0.82
18	540	0.99	0.35	0.62	0.70	0.82
19	570	0.99	0.35	0.62	0.70	0.82
20	600	0.99	0.35	0.62	0.70	0.82

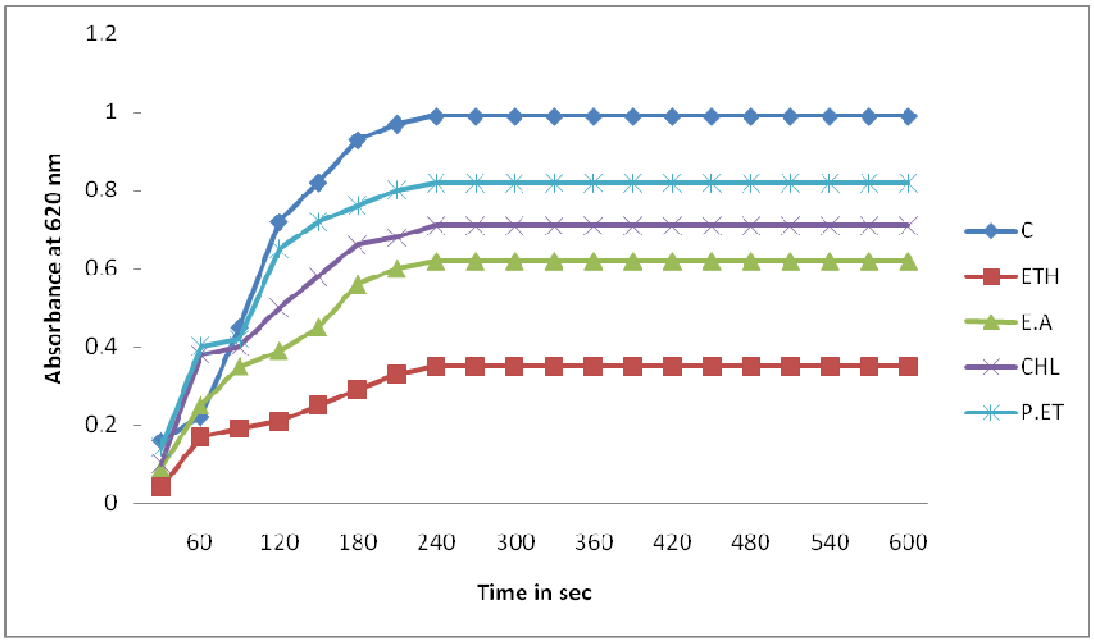


Fig 5.1 Calcium oxalate crystallization inhibition with plant extract

The graph shows an initial detectable increase in the turbidity after induction of the crystallization with sodium oxalate, was observed. In the control experiment, there was an initial steep rise in turbidity (the nucleation phase), on attaining its maximum, it was followed by a decrease (the aggregation phase).

The various plant extract, inhibited the slope of turbidity followed by very slow decrease in graph. Crystallization was inhibited significantly with ethanolic plant extract followed by ethyl acetate, chloroform and petroleum ether plant extract.

The linear proportion of the graph was taken up for detecting the slope.

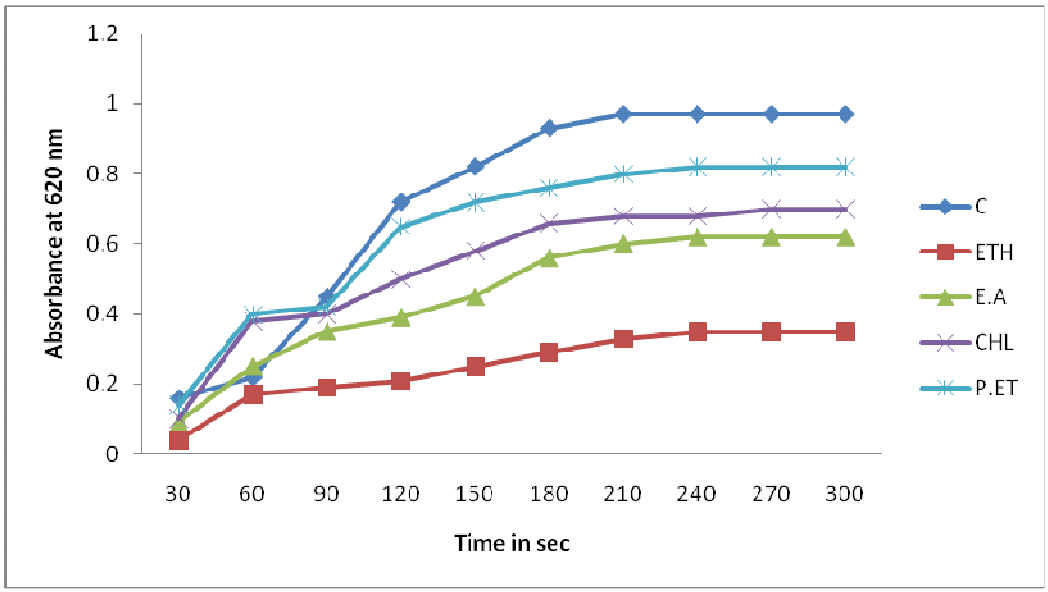
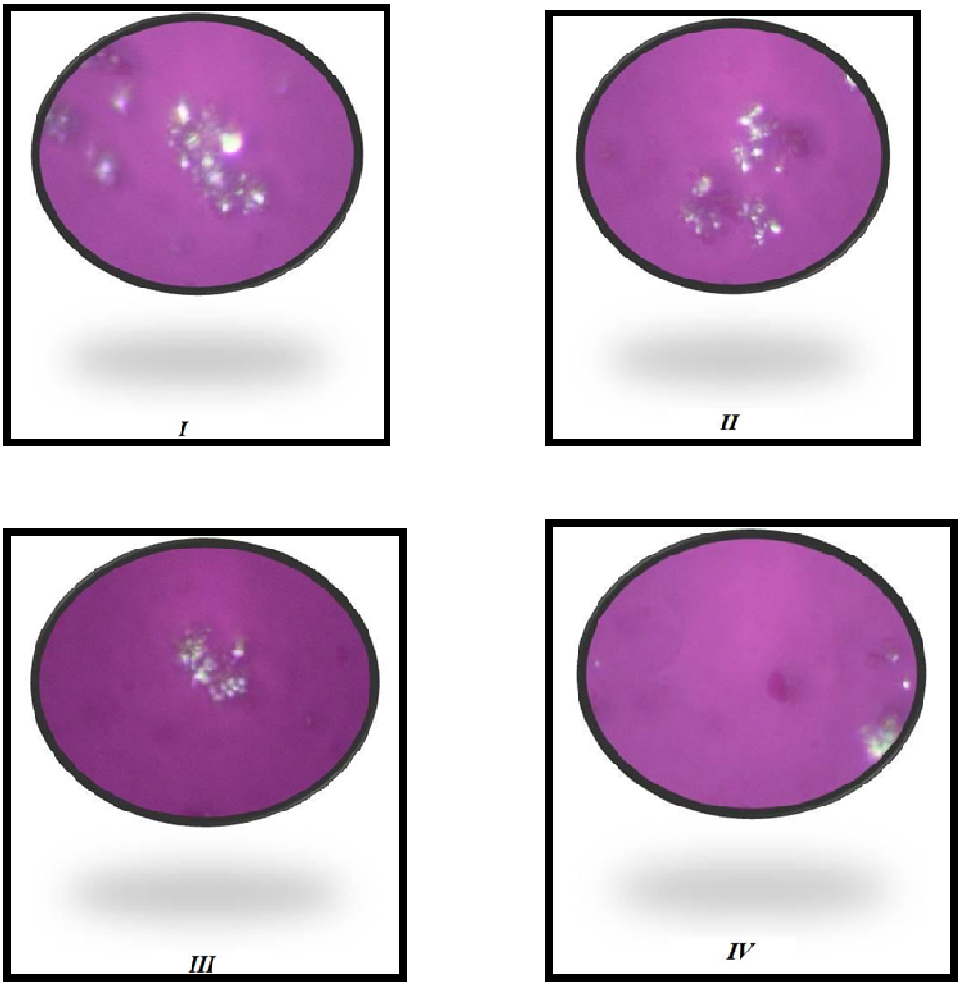


Fig 5.2 Linear portion of the graph

MICROSCOPIC STUDY:

The ethanolic plant extract on being examined under the microscope show that the ethanolic plant extract had decreased the number of crystals. The first photograph corresponding to the stages of the growth and aggregation for the crystallization without inhibitor of calcium oxalate. The comparison of photographs (II,III,IV and V) of the tries with inhibitor (ethanolic extract) enabled us to understand that crystals were greatly reduced in number, which explains that the ethanolic extract produces significant quantity of inhibition on growth of the crystal.

S.No	Extracts	% Inhibition
1.	Ethanolic extract	82%
2.	Ethyl acetate extract	75%
3.	Chloroform extract	66%
4.	Pet. ether extract	55%



I **Control**

II, III, IV **With Inhibitor solution in time dependent manner shows decrease in crystal number after 10min, 20 min, 30 min**

Fig 5.3 Microscopic photographs of calcium oxalate crystallization inhibition

The photograph clearly indicates that in case where the ethanolic plant extract was not added, the number of crystals is maximum (photo I). In the photographs II, III and IV which

represent the crystal formation when the solution was mixed with plant extract , there was a clear indication that there is a dose dependent manner of crystals formed and with time.

Kidney oxalate stone is the result of super saturation of urine with certain urinary salts such as calcium oxalate. Since crystallizable oxalate species are pH dependent, the crystallization of oxalate in the absence of an inhibitor, led to the formation of calcium oxalate monohydrate monitored by light microscope, the process of calcium oxalate crystallization in control without the addition of inhibitors is shown in (Figure I).

In the crystal growth experiments shown nucleation, growth and aggregation, the rate of crystallization is usually controlled by the number of crystals of calcium oxalate as a function of time, following the introduction of seed crystals. Entitled constant volume against time in the composition calcium oxalate experiments determined that the rate of growth of crystals were made in the absence and presence of ethanolic plant extract.

In order to assess the inhibiting potential of substances for oxalate crystallization and understand the mechanisms of action of these inhibitors on oxalate crystallization steps viz. nucleation, growth, aggregation, we tested the effectiveness of ethanolic plant extract.

5.2 *IN-VIVO* ANTIUROLITHIATIC ACTIVITY OF VARIOUS EXTRACT OF WHOLE PLANT OF *Azima tetracantha* Lam. ON ETHYLENE GLYCOL INDUCED IN RATS

From the acute toxicity study, the LD_{50} cut-off dose was found to be 2000 mg/kg body weight for the plant *Azima tetracantha* Lam. Hence the therapeutic dose was taken as 200 mg/kg/b.w. for the plant.

Ethylene glycol (EG) is rapidly absorbed and metabolized in the liver via alcohol dehydrogenase/ aldehyde dehydrogenase to glycolic acid. Glycolic acid is oxidized to

glycolate, which, in turn, is further oxidized to oxalic acid by glycolate oxidase. High doses of EG (>2,500 mg/kg body wt.), particularly when given as an oral bolus, cause the saturation dependent accumulation of glycolic acid in the plasma so glycolate oxidase (GO) is one of the rate limiting enzymes in the metabolism of EG (Green et al.,2005).

Table5.2Estimation of urine volume

Group	Volume of urine(ml)
Control	2.08±0.15
Induced (Ethylene glycol 0.75%)	1.18±0.14
Standard (Cystone 750mg/kg)	2.5±0.14
Ethanolic plant extract (200mg/kg)	2.1±0.13
Ethyl acetate plant extract (200mg/kg)	2.06±0.13
Chloroform plant extract (200mg/kg)	1.9±0.17
Pet.ether plant extract (200mg/kg)	1.16±0.18

The values are expressed as Mean ± SD, n=6.

*P < 0.05 as compared to control.

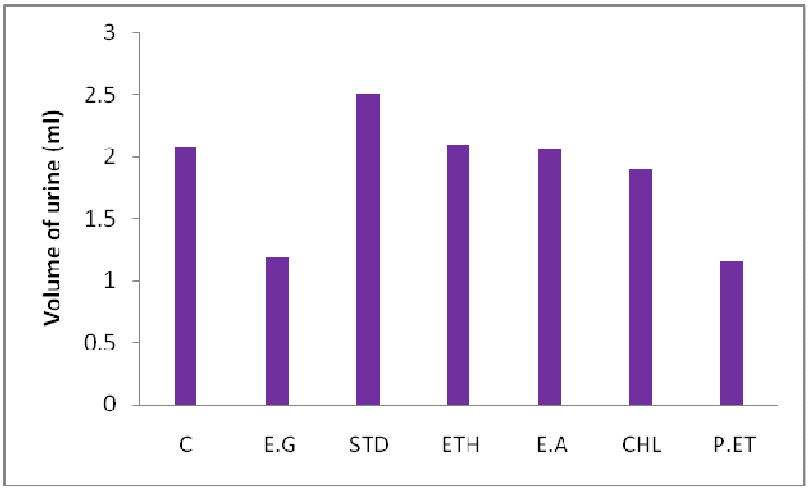


Fig 5.4 Estimation of Urine Volume

The glomerular filtration rate (GFR) is an important parameter for ensuring renal function and it gets decreased in urolithiasis due to the obstruction to the outflow of the urine by stones in urinary system,

Which leads to a rise in nitrogenous waste products like urea, creatinine and uric acid in blood.

Table 5.3 pH of Urine

Group	pH
Control	7.1 ± 0.16
Induced (Ethylene glycol 0.75%v/v)	6.51 ± 0.24
Standard (Cystone 750mg/kg)	7.5 ± 0.08
Ethanolic plant extract (200mg/kg)	7.15 ± 0.16
Ethyl acetate plant extract (200mg/kg)	7.0 ± 0.08
Chloroform plant extract (200mg/kg)	7.0 ± 0.08
Pet.ether plant extract (200mg/kg)	6.85 ± 0.05

The values are expressed as Mean ± SD, n=6.

*P < 0.05 as compared to control.

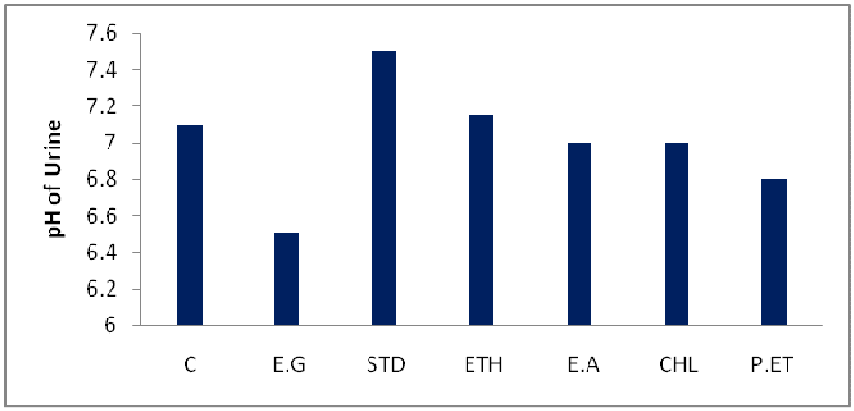


Fig 5.5 Estimation of Urine pH

Urinary pH of control is neutral, ethylene glycol induced rats pH is reduced compared with control group. Treatment with (Cystone 750 mg/kg) was found to increase the urine pH (7.5±0.08), whereas, groups receiving plant extract also found to increase the urinary pH in a dose dependent manner but ethanol and ethyl acetate plant extract produces significant increase in pH nearly to that of control ($p<0.05$).

Table 5.4 Estimation of calcium, magnesium, phosphorous and Oxalate

Group	Calcium (mg/dl)	Magnesium (mg/dl)	Oxalate (mg/dl)	Phosphorus (mg/dl)
Control	8.56 ± 0.19	2.63 ± 0.08	1.36 ± 0.10	5.36 ± 0.10
Induced(Ethylene glycol 0.75%v/v)	11.6 ± 0.34	1.33 ± 0.12	5.5 ± 0.37	7.71 ± 0.17
Standard (Cystone 750mg/kg)	8.88 ±0.07	2.66 ± 0.22	1.44 ± 0.08	5.86 ± 0.05
Ethanolic plant extract (200mg/kg)	8.53 ± 0.13	2.66 ± 0.16	1.63 ± 0.08	5.46 ± 0.12
Ethyl acetate plant extract (200mg/kg)	8.7 ± 0.20	2.4 ± 0.23	1.88 ± 0.07	5.95 ± 0.25
Chloroform plant extract (200mg/kg)	9.06 ± 0.12	2.21 ± 0.17	2.43 ± 0.17	6.1 ± 0.24
Pet.ether plant extract (200mg/kg)	10.11 ± 0.48	1.76 ± 0.16	2.71 ± 0.11	6.7 ± 0.31

The values are expressed as Mean ± SD, n=6.

*P < 0.05 as compared to control.

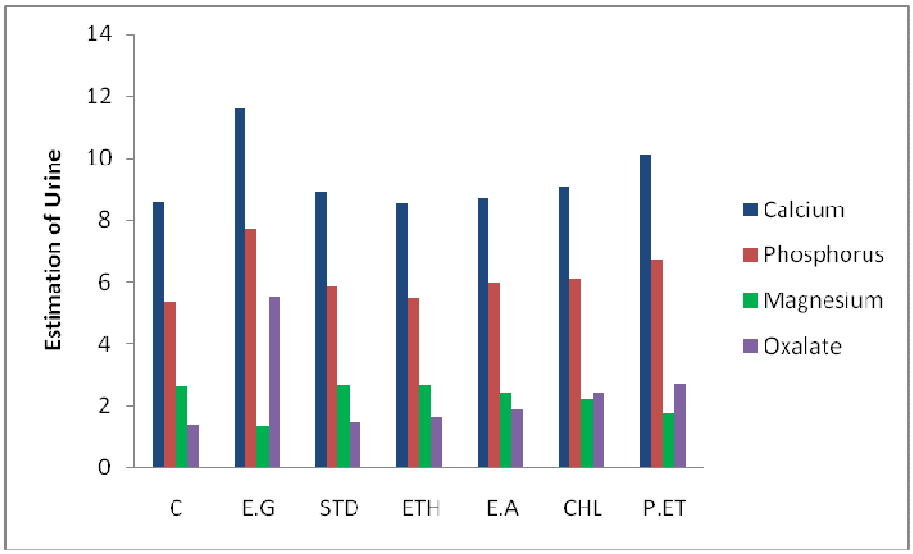


Fig 5.6 Estimation of Ca,PO_4, Mg and Oxalate

Formation of calculi is associated with super saturation of urine with stone forming constituents. Repeated administration of ethylene glycol (0.75%v/v) cause generation of kidney stone and the most important cause for it was found to be presence of calcium oxalate. Increase in the urinary concentration of oxalate is considered as one of the major cause responsible for formation of stone. Stone formation in ethylene glycol administered animals is caused by hyperoxaluria, which enhances renal retention and excretion of oxalate[50]. In this study it was observed that ethanol and ethyl acetate plant extract (200mg/kg) significantly decreased level of oxalate, calcium, phosphate and increased the level of magnesium in urine.

The urine mineral constituents-calcium, magnesium, oxalate and phosphorus in control and experimental rats. Calcium, oxalate and phosphorus play a vital role in renal calculogenesis. In the present study, chronic administration of 0.75% v/v ethylene glycol aqueous solution for 28 days resulted in hypercalciuria in rats[64]. Phosphorus, oxalate and calcium excretion were significantly increased ($p<0.05$), whereas magnesium decreased in urine and kidney of EG treated animals group II as compared to group I.

However, supplementation with plant extracts at 200 mg/kg body weight and cystone 750 mg/kg body weight significantly ($P < 0.05$) lowered the elevated level of phosphorus and calcium in urine as compared to the group II animals.

Magnesium level in the standard and test groups came close to normal and was comparable to the levels in the belonging to the untreated group II.

However among the four plant extracts the ethanolic plant extract and ethyl acetate plant extract produces significant effect.

Table 5.5 Estimation of serum constituents

Group	Urea(mg/dl)	Uric acid(mg/dl)
Control	9.9 ± 0.07	2.21 ± 0.17
Induced (Ethylene glycol 0.75% v/v)	15.73 ± 0.18	4.78 ± 0.22
Standard (Cystone 750mg/kg)	10.15 ± 0.09	2.33 ± 0.19
Ethanolic plant extract (200mg/kg)	9.81 ± 0.16	2.43 ± 0.13
Ethyl acetate plant extract (200mg/kg)	10.4 ± 0.10	2.73 ± 0.19
Chloroform plant extract (200mg/kg)	10.86 ± 0.05	3.05 ± 0.12
Pet.ether plant extract (200mg/kg)	11.28 ± 0.14	3.46 ± 0.13

The values are expressed as Mean ± SD, n=6.

*$P < 0.05$ as compared to control

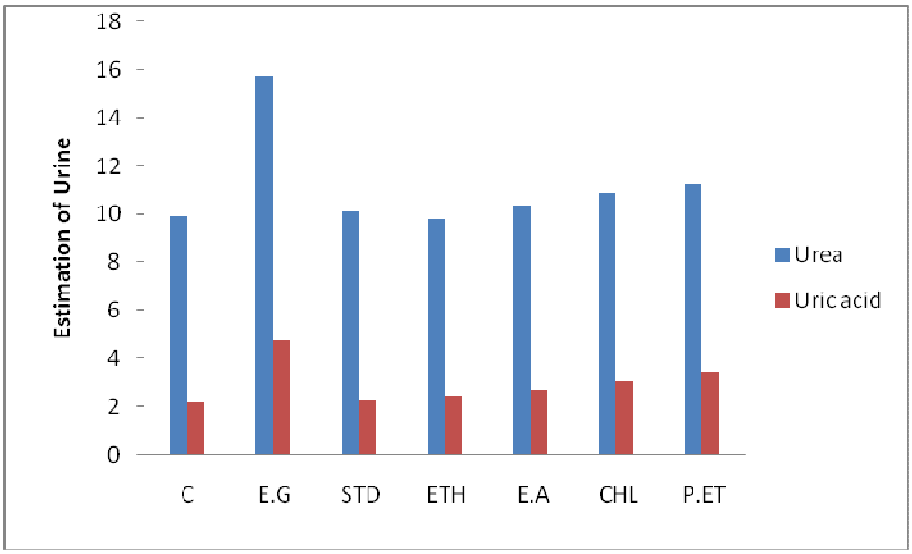


Fig 5.7 Estimation of Urea and Uric acid

Table 5.6 Estimation of creatinine and BUN

Group	Creatinine(mg/dl)	BUN(mg/dl)
Control	0.38 ± 0.02	4.70 ± 0.05
Induced (Ethylene glycol 0.75%v/v)	0.64 ± 0.03	7.5 ± 0.15
Standard (Cystone 750mg/kg)	0.42 ± 0.04	4.79 ± 0.21
Ethanolic plant extract (200mg/kg)	0.42 ± 0.03	4.61 ± 0.09
Ethyl acetate plant extract (200mg/kg)	0.48 ± 0.05	4.93 ± 0.18
Chloroform plant extract (200mg/kg)	0.51 ± 0.02	4.98 ± 0.20
Pet.ether plant extract (200mg/kg)	0.58 ± 0.04	5.47 ± 0.15

The values are expressed as Mean ± SD, n=6.

*P < 0.05 as compared to control

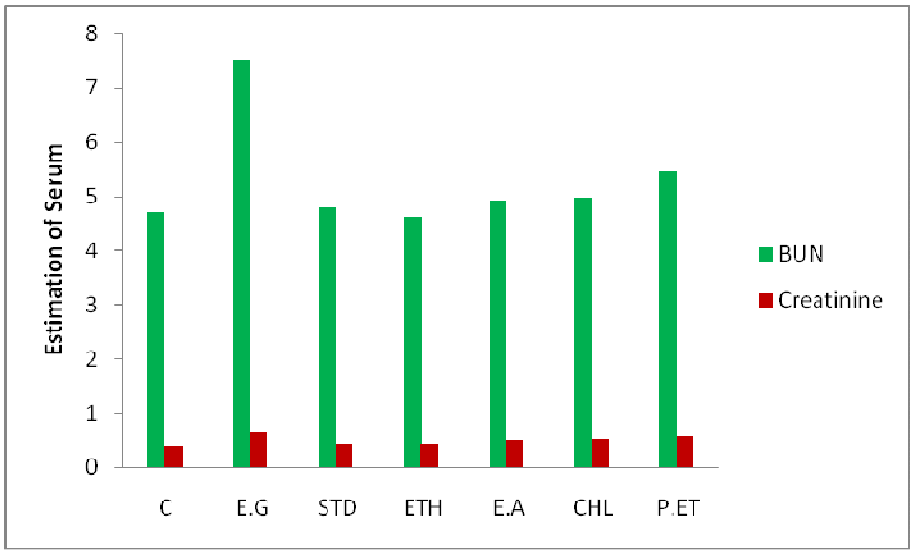


Fig 5.8 Estimation of creatinine and BUN

Decrease in glomerular filtration due to obstruction generated in the kidney cause accumulation of waste products in blood, thus level of waste components like BUN, uric acid, urea and creatinine increases in blood.

In calculi-induced rats (Group II), marked renal damage was seen by the elevated serum levels of creatinine, uric acid, urea and Blood Urea Nitrogen (BUN)[50]. However, treatment with plant extract restored the serum levels of creatinine, uric acid and BUN.

The ethanolic and ethyl acetate plant extract (Group IV and V) and Cystone (Group III) significantly ($p<0.05$) lowered the elevated serum level uric acid as compared to group I.

Chloroform and pet.ether extract also decreased the level of urea, uric acid, creatinine and BUN level in the serum of urolithiatic induced rats.

Table 5.7 Estimation of ACP, AST, ALP and ALT in serum

Group	ACP (U/ml)	AST (U/ml)	ALP (U/ml)	ALT (U/ml)
Control	66.6 ± 1.36	32.6 ± 1.63	73.3 ± 1.63	31 ± 0.89
Induced (Ethylene glycol 0.75%)	113.8 ± 3.06	87.5 ± 4.32	153.6 ± 2.8	95.1 ± 0.75
Standard (Cystone 750mg/kg)	73 ± 1.67	37.1 ± 1.60	75.6 ± 1.96	32.6 ± 2.06
Ethanolic plant extract (200mg/kg)	71.6 ± 2.73	37.8 ± 1.32	76 ± 2.52	35 ± 1.41
Ethyl acetate plant extract (200mg/kg)	77 ± 2.09	40.3 ± 1.36	79.6 ± 1.50	42.1 ± 2.22
Chloroform plant extract (200mg/kg)	81 ± 0.89	43.6 ± 1.50	88.3 ± 1.50	41.8 ± 1.72
Pet.ether plant extract (200mg/kg)	87.1 ± 2.78	51.8 ± 2.31	98 ± 1.78	50.8 ± 3.48

The values are expressed as Mean ± SD, n=6.

*P < 0.05 as compared to control.

From the table it was evident that the levels of the enzymes like ACP, AST, ALP and ALT were significantly increased ($p<0.05$) in serum of ethylene glycol intoxicated (group II) rats[64]. In the ethanolic (group IV) and ethyl acetate (group V) plant extract treated groups the enzymes level were decreased significantly in the serum when compared to group II rats. As the evident from the above results, the ethanolic and ethyl acetate extract of *Azima tetracantha* Lam. seems to contain antiurolithiatic activity. However, when the standard drug, Cystone treated rats (group III) were compared with plant extract treated rats (group IV&V), there is no significant difference ($p<0.05$) was observed.

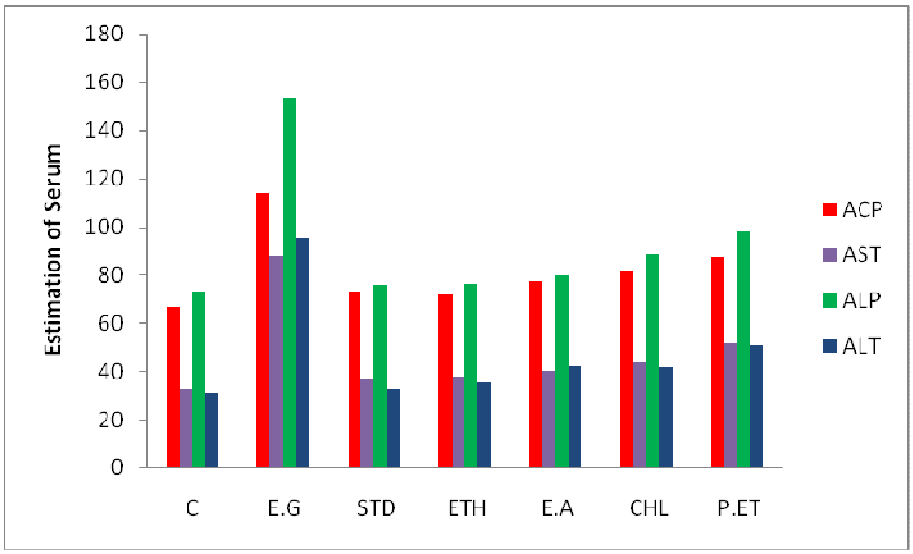


Fig 5.9 Estimation of ACP, AST, ALP and ALT in serum

Table 5.8 Estimation of ACP, AST, ALP and ALT in kidney

Group	ACP	AST	ALP	ALT
Control	12.5 ± 0.87	11.7 ± 0.70	6.66 ± 0.51	13.01 ± 0.33
Induced (Ethylene glycol 0.75%v/v)	8.5 ± 0.44	6.78 ± 0.51	3.5 ± 0.35	9.48 ± 0.33
Standard (Cystone 750mg/kg)	13.2 ± 0.87	12 ± 0.89	6.67 ± 0.24	12.83 ± 0.26
Ethanolic plant extract (200mg/kg)	13.1 ± 0.83	11.9 ± 0.93	6.51 ± 0.31	12.81 ± 0.26
Ethyl acetate plant extract (200mg/kg)	11.3 ± 0.85	10.6 ± 0.45	6.15 ± 0.28	11.9 ± 0.08
Chloroform plant extract (200mg/kg)	10.6 ± 0.58	9.7 ± 0.38	5.5 ± 0.32	11.3 ± 0.32
Pet.ether plant extract (200mg/kg)	9.8 ± 0.68	8.66 ± 0.40	4.8 ± 0.20	10.73 ± 0.47

The values are expressed as Mean ± SD, n=6.

*P < 0.05 as compared to control.

Units:

ALP, ACP -µ moles of phenol liberated/min./mg of protein

AST, ALT - µ moles of pyruvate liberated/min./mg of protein

LDH - µ moles of pyruvate liberated/L

From the table it was evident that the levels of the enzymes like ACP, AST, ALP and ALT were significantly decreased ($p<0.05$) in kidney of ethylene glycol intoxicated (group II) rats[64]. In the ethanolic (group IV) and ethyl acetate (group V) plant extract treated groups the enzymes level were increased significantly in the serum when compared to group II rats. As the evident from the above results, the ethanolic and ethyl acetate extract of *Azima tetracantha* Lam. seems to contain antiurolithiatic activity. Chloroform and Pet. Ether extract also produces anti-urolithiatic activity but not as much as ethanol and ethyl acetate extract. However, when the standard drug, Cystone treated rats (group III) were compared with plant extract treated rats (group IV&V), there is no significant difference ($p<0.05$) was observed.

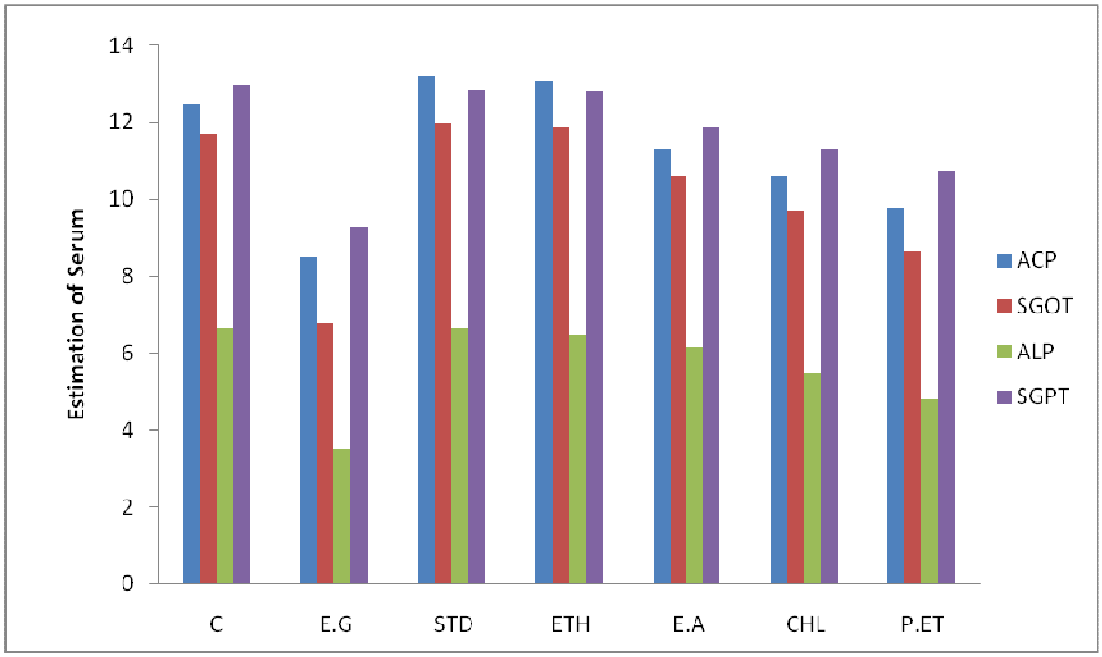


Fig 5.10 Estimation of ACP, SGOT, ALP and SGPT in kidney

Table 5.9 Estimation of LDH in Serum and Kidney

Group	LDH in Serum (µmoles/L)	LDH in Kidney (U/mg)
Control	**145.9 ± 0.70**	**3.71 ± 0.02**
Induced (Ethylene glycol 0.75%)	**205.4 ± 0.49**	**7.58 ± 0.07**
Standard (Cystone 750 mg/kg)	**146.8 ± ± 0.67**	**3.77 ± 0.02**
Ethanolic plant extract (200 mg/kg)	**147.9 ± 0.50**	**3.74 ± 0.03**
Ethyl acetate plant extract (200 mg/kg)	**152 ± 0.40**	**4.18 ± 0.11**
Chloroform plant extract (200 mg/kg)	**154.5 ± 0.8**	**4.5 ± 0.12**
Pet.ether plant extract (200mg/kg)	**160.75 ± 0.27**	**4.9 ± 0.16**

The values are as Mean ± SD, n=6.

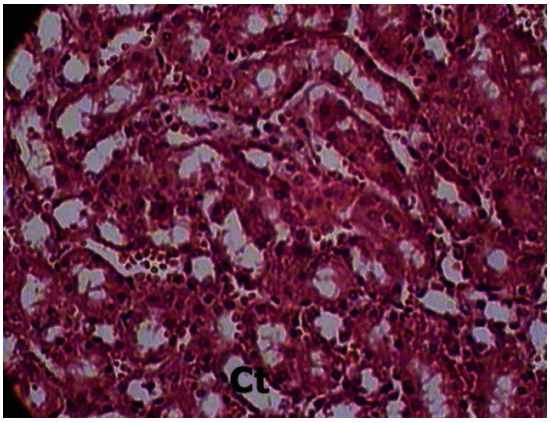
*P < 0.05 as compared to control.

The above table represents the levels of lactate dehydrogenase levels in the serum and kidney homogenate. Level of LDH was significantly increased in serum and kidney homogenate of ethylene glycol intoxicated (group II) rats. LDH is an oxalate synthesizing enzyme; its activity was increased on ethylene glycol administration[65]. It was released into the blood stream and urine. This may be attributed to oxalate induced renal and hepatic cellular damage. Renal damage is particularly confined to the proximal tubule, a part of the nephron closely involved in handling urinary oxalate. The treatment (groups IV&V) with ethanolic and ethyl acetate plant extract after ethylene glycol induction showed a significant ($p<0.05$) decrease in the activity of these enzymes in serum and tissue homogenate when compared to group II rats. As evident from above results, the plant *Azima tetracantha* Lam.

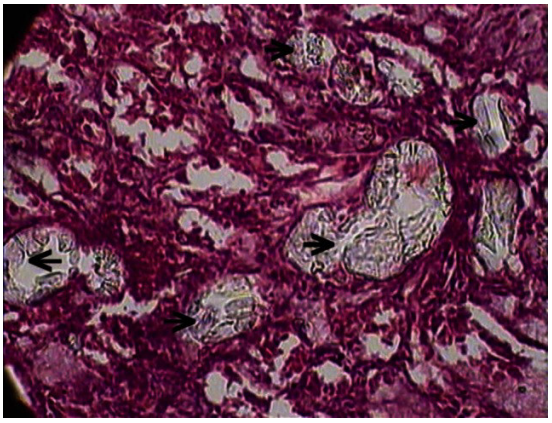
seems to contain antiurolithiatic activity. However, when the standard drug, Cystone treated rats (group III) were compared with plant extract administered group (group IV&V), no significant difference ($p<0.05$) was observed between these three groups of animals.

HISTOPATHOLOGICAL EXAMINATION OF THE RAT KIDNEYS

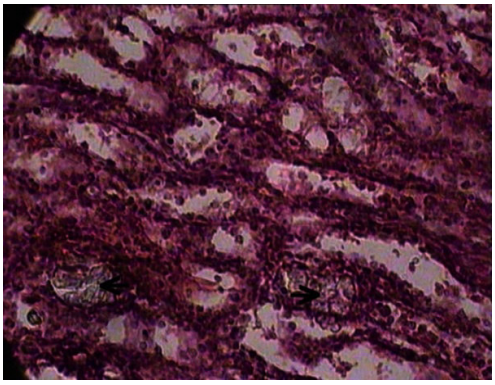
The present histopathological studies showed EG exposed urolithiatic rats showed deposition of calcium oxalate crystals in lumen of Proximal convoluted tubule Figure-1. Tubules were highly dilated and cystic with sloughing off the tubular epithelial cells. While in plant extract treated animals, it apparently retained normal morphological and microscopical anatomical architecture of the nephrons. However, mild degeneration of cells and cyst formation was observed.



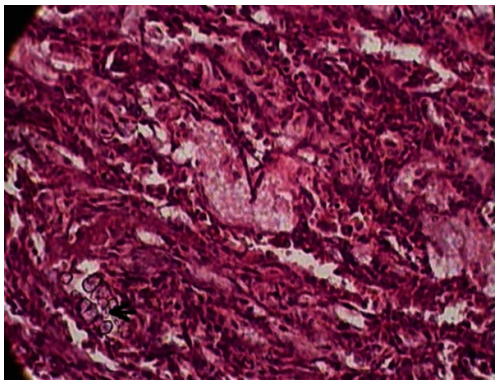
Group I: Control rat



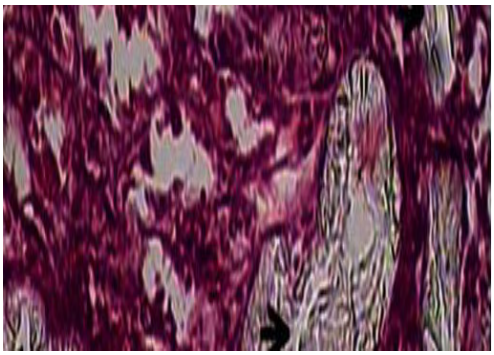
Group II: Ethylene glycol induced rat



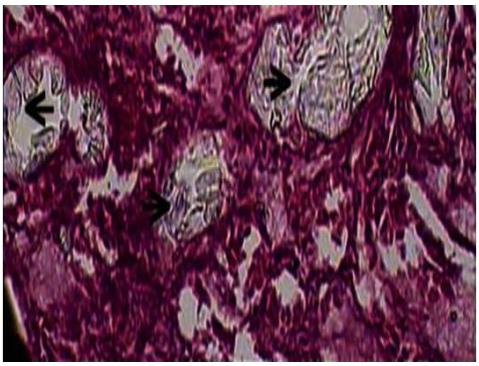
Group III: Standard Cystone treated rat



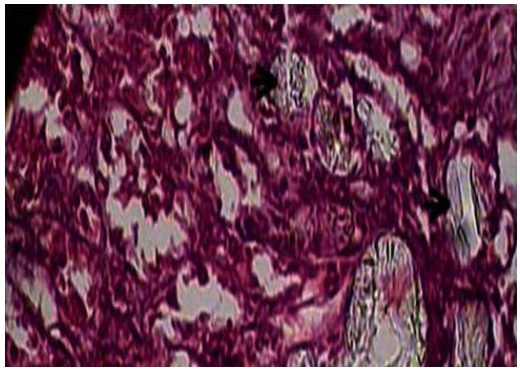
Group IV: Ethanolic extract treated rat



Group V: Ethyl acetate extract treated rat



Group VI: Chloroform extract treated rat



Group VII: Pet. Ether extract treated rat

The antiurolithiatic effect was further confirmed by kidney histopathological studies. Indeed, kidney sections of untreated rat showed abundant crystal depositions. Furthermore, renal epithelial cells had more tubular dilatation and damage shown by large spaces in the tissue. In treated rats, less crystal depositions were seen compared to untreated animals and the necrosis as well as the tubule dilatation was very limited. Renal stone deposition damages the renal tissue and detoriate the renal function. Lithogenic treatment caused impairment of renal functions of the untreated rats as evident from the markers of glomerular and tubular damage: raised BUN, uric acid, urea and serum creatinine that was lowered in animals receiving plant extract.

Tissue injury and inflammation in these animals is due to exposure to phosphate and calcium phosphate crystals, leading to the generation of reactive oxygen species, development of oxidative stress, lipid peroxidation and depletion of antioxidant enzymes. Renal epithelial injury further promotes crystal retention, as epithelial injury exposes a variety of crystal adhesion molecules on epithelial surfaces and promotes stone formation. Probably antioxidant constituents of the plant restore the renal antioxidant enzyme and prevent renal cell injury.

CONCLUSION

- From the present study we conclude that the preliminary phytochemical analysis of *Azima tetracantha* Lam. indicated the presence of Alkaloids, Flavonoids, Proteins, Saponins, Terpenoids, Phytosterols, Carbohydrates and Fatty acids.

- *In-vitro* Calcium oxalate crystallization inhibition study was evaluated. From this study we conclude that the ethanol, ethyl acetate, chloroform and pet.ether extracts of *Azima tetracantha* Lam. inhibits the calcium oxalate crystallization in the order of 82%, 75%,66% and 55% respectively.

- *In-vivo* Anti urolithiatic activity of various extracts (Ethanol, Ethyl acetate, Chloroform and Pet.ether) of *Azima tetracantha* Lam. was evaluated and we conclude that the ethanol and ethyl acetate extracts of the plant *Azima tetracantha* Lam. produced significant antiurolithiatic activity like that of standard. Moderate effect was produced by chloroform extract.

LIST OF ABBREVIATIONS

WHO	--	World Health Organisation
AT	--	*Azima tetracantha* Lam.
E.coli	--	Escherichia coli
C	--	Control
E.G	--	Ethylene glycol
STD	--	Standard (Cystone)
ETH	--	Ethanol
E.A	--	Ethyl acetate
CHL	--	Chloroform
P.ET	--	Petroleum ether
Pet.ether	--	Petroleum ether
LDH	--	Lactate dehydrogenase
MDH	--	Malate dehydrogenase
AST	--	Aspartate aminotransferase
HEDTA	--	Hydroxyl Ethylethylene Diamine Triactic Acid
NPP	--	Non-prostatic phosphatase
NADP	--	Nicotinamide adenine dinucleotide phosphate
CPCSEA	--	Committee for the Purpose of Control and Supervision on Experiment on Animals
ALT	--	Alanine Aminotransferase
AST	--	Aspartate Aminotransferase
FDA	--	Food and Drug Administration
Rtd	--	Retired
CSIR	--	Council for Scientific and Industrial Research
EDTA	--	Ethylene diamine tetra acetic acid

ATP	--	Adenosine Tri Phosphate
NAD	--	Nicotinamide Adenine Dinucleotide
NADH	--	Reduced Nicotinamide Adenine Dinucletide
IAEC	--	Institutional Animal Ethical Committee
OECD	--	Organisation for Economic Co-operation and Development
SGOT	--	Serum Glutamate Oxaloacetic Transaminase
SAP	--	Serum Alkaline Phosphate
SGPT	--	Serum Glutamic Pyruvic Transaminase
WBC	--	White Blood Cell
H_2O_2	--	Hydrogn Peroxide
H_2O	--	Water
ALP	--	Alkaline Phosphatase
ACP	--	Acid Phosphatase
ANOVA	--	Analysis of Variance
SD	--	Standard Deviation
Ppm	--	parts per million
rpm	--	Revolutions per minute
mins	--	Minutes

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