EVALUATION OF *IN VITRO* AND *IN VIVO* ANTI UROLITHIATIC ACTIVITY OF *Justicia Tranquebariensis* ON MALE WISTAR RATS

A dissertation submitted to

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IN

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Submitted by

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MAY-2019

CERTIFICATE

This is to certify that the dissertation entitled **" EVALUATION OF *IN VITRO* AND *IN VIVO* ANTI UROLITHIATIC ACTIVITY OF *Justicia Tranquebariensis* ON MALE WISTAR RATS"** submitted by **Reg. No.261726054** in partial fulfillment of the requirements for the award of degree in master of pharmacy in pharmacology by The Tamil Nadu Dr. M.G.R. Medical university, Chennai, is a bonafide work done by his during the academic year 2018-2019 under the guidance of **Dr.M.SAKTHI ABIRAMI, M.Pharm., Ph.D.,** Assistant Professor in Pharmacology, Institute of Pharmacology, Madras Medical College, Chennai-600 003.

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LIST OF ABBREVIATIONS

UTI	Urinary Tract Infection
IVU	Intravenous Urogram
KUB	Kidney UreteroBlader
ESWL	Extracorporeal Shock Wave Lithotripsy
WHO	World Health Oraganisation
IAEC	Institutional Animal Ethical Committee
OECD	Organisation for Economic Co-operation and Development
BUN	Blood Urea Nitrogen
PAP	Peroxidise –Antiperoxidase
SEM	Standard error for the mean
ALT	Alanine Amino Transferase
AST	Aspartate amino transferase
GO	Glycolate Oxidase
JT	*Justicia tranquebariensis*
GFR	Glomerular Filtration Rate
CPCSEA	Committee For The Purpose of Control and Supervision of Experiments on Animals

1. INDRODUCTION

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 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 introduction of new therapeutic agents.

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[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. Urolithiasis formation is a multifactorial processwhich 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 glycosaminoglycan 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.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.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 are the most commonly experience their first episode between 20-30 years of age, while for women the age at first presentation is somewhat later[11].

Nomenclature of Stones

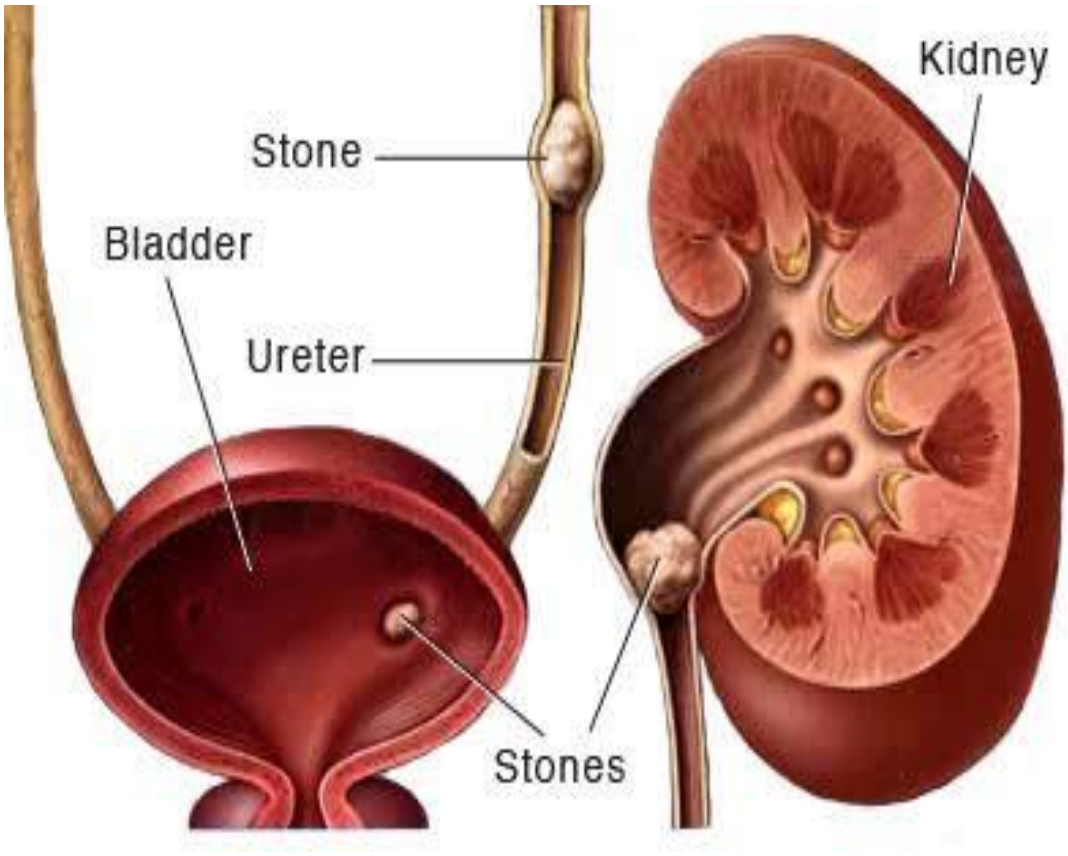
The word "crystal" is derived from the Greek work krystallosus, which means "ice" and is used to refer to the solid phase of substances having a specific internal structure and enclosed by symmetrically arranged planar surfaces.

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[12]. Kidney 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[13].

Nephrolithiasis and ureterolithiasisa kidney stone, also known as a renal calculus. It is a solid concretion or crystal aggregation formed in the kidneys from dietary minerals in the urine. Kidney stonestypically leave the body by passage in the urine stream, and many stones are formed andpassed without causing symptoms. If stones grow to sufficient size (usually at least3millimeters (0.12n)) they can cause obstruction of the ureter. Ureteral obstruction causespostrenalazotemiaand hydronephrosis(distension and dilation of the renalpelvis 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.

Fig.1: Different location of stones in kidney



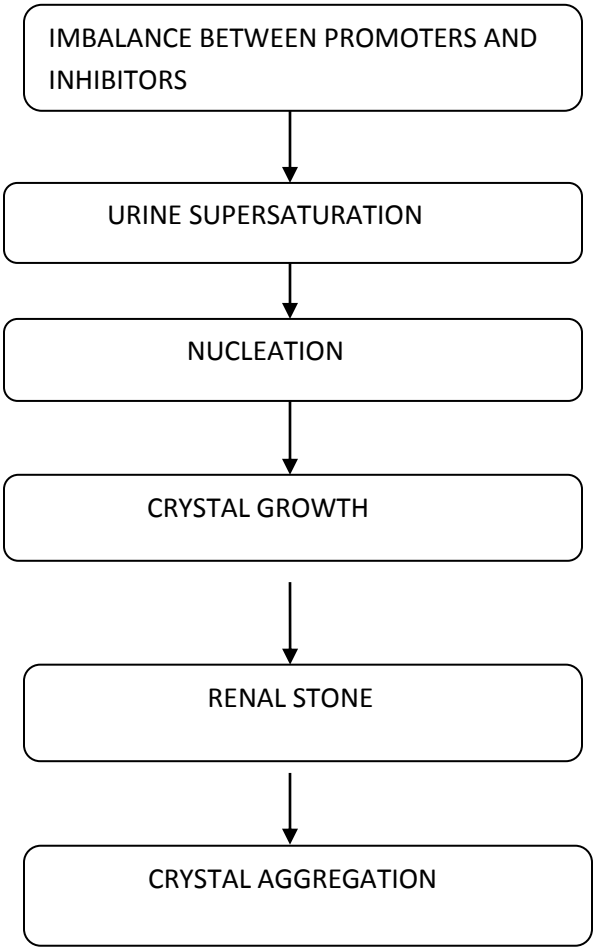
ETIOLOGY AND PRECIPITATING FACTORS

- 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[14].

PATHOGENESIS OF RENAL STONE FORMATION

The physical process of stone formation is a complex cascade of events, result from the growth of crystals leads to stones formation[15]. The process of stone formation is depend on volume of urine, comprise concentrations of calcium, phosphate, oxalate and sodium ions[16]. High levels of ionic concentration, low urinary volume, low pH, and low citrate levels privilege the formation of urinary calculi. The pathogenesis of urinary calculi formation is the end result of the fundamental multi-step physicochemical processes. The genetic, metabolic, environmental and dietetic factors are involved in the pathogenesis of urolithiasis, all of them privilege the crystallization of salts, formed in inside renal tubules. Crystalluria is often observed in normal individual, but if crystals remain apart from each other. They are washed away by urine flow; however, some chemical and electrical forces trigger the process of aggregation. The crystals aggregate and attaches to epithelium, which allows them to growing and forming the stones[17].



Nucleation

Nucleation is the formation of a solid crystal phase in a solution. The stone formation starts from the nuclei, which means the process of new crystal formation. It is an essential step in renal stone formation the term super saturation refers to a solution that contains more of the dissolved material than could be dissolved by the solvent under normal circumstances. Crystal nucleation is the first step in the formation of stone which can either be homogeneous nucleation of a salt occurs in unstable zone of super saturation. Crystalluria and stone formation seem to be the result of hetrogeneous nucleation induced by promoters. Promoters probably present in preformed surfaces that reduce the surface energy required for crystallisation. During crystal growth, the

free energy of solution continues to decrease as new crystal components are taken from the solution and become part of the crystal structure. Once formed, the crystalline particles can bind to each other in either an oriented or random growth pattern and then grow into a larger particle[18].

Crystal growth

After the nucleation process, the micro crystals can mature by epitaxially mediated crystal growth. Epitaxy is oriented overgrowth of one crystalline material on to a substrate crystalline lattice. Monoepitaxial growth refers to the adsorption of the molecules or ions one by one on the crystal surface from supersaturated urine and heteroepitaxial growth refers to direct growth of one crystal on a surface of different composition and the surfaces of crystal and substrate.

Several atoms or molecules in a super-saturated liquid start forming clusters. The total free energy of the cluster is increased by the surface energy; however, this is significant only when the cluster is small. Crystal growth is determined by the molecular size and shape of the molecule, the physical properties of the material, pH, and defects that may form in the crystal's structure. Crystal growth is one of the prerequisites for particle formation[19].

Aggregation

Aggregation is a process in which crystal nuclei bind to each other to form larger particles. The initial nuclei can grow by further addition of desired salts. A small inter-particle distance increases the attractive force and privileges particle aggregation. Crystal aggregation plays an important role in stone formation. In various steps of stone formation, crystal aggregation is a more significant step and then nucleation and growth. Aggregation of particle in solution is

determined by a balance of forces, between aggregating effects and disaggregation effects and also a small inter particle distance that privileges particle aggregation[20].

Retention

Crystal retention can be caused by the association of crystals with the epithelial cells lining. Urolithiasis requires formation of crystals followed by their retention and accumulation in the kidney. Another process that may lead to stone formation is crystal retention. i.e., crystal precipitation, growth, and aggregation, which results in urinary stone formation, if the nucleated crystals were flushed out by urinary flow. Retention might also depend on the composition of the renal tubular epithelial cell surface[21].

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).

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.

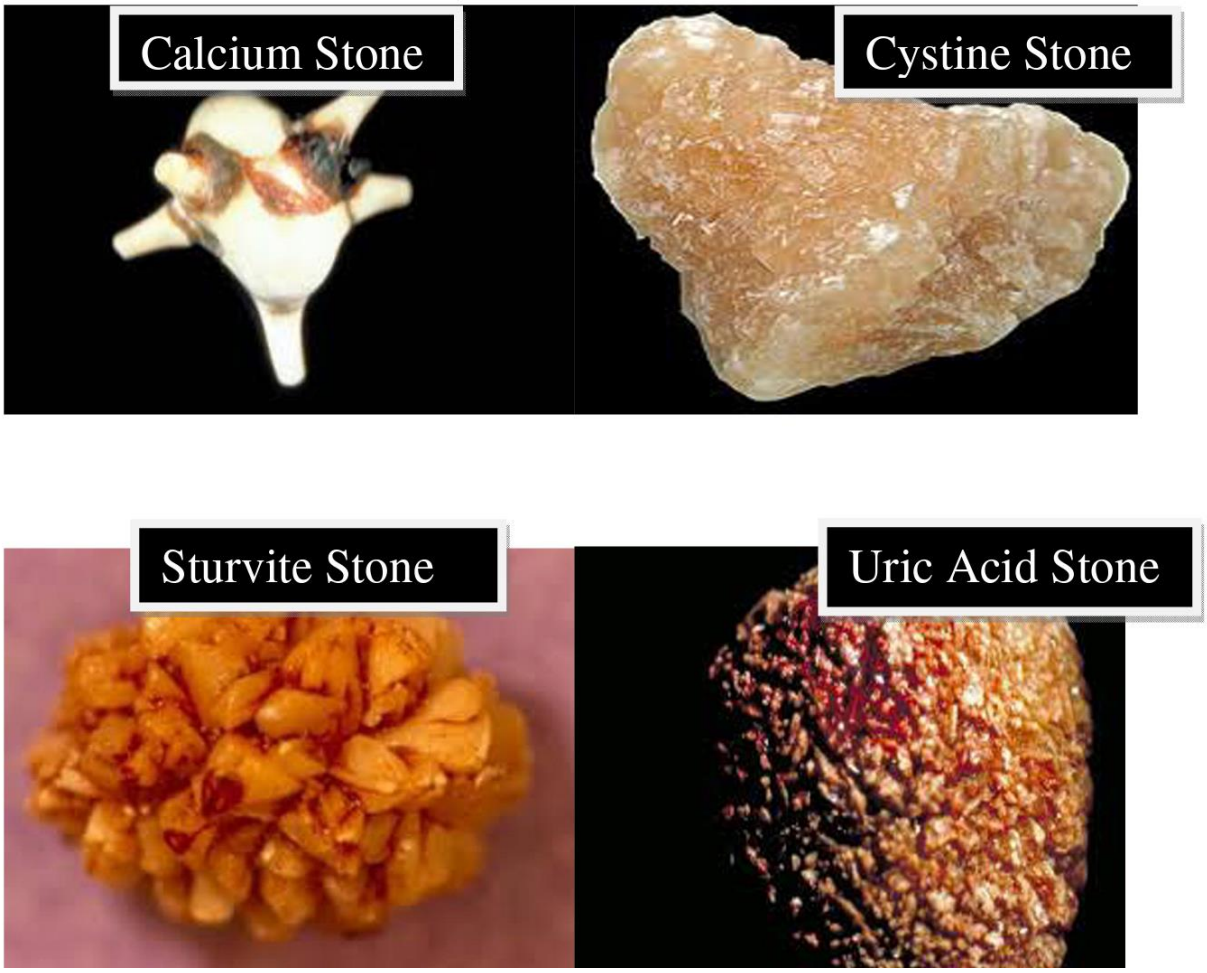
Cystine stonescan form in people who have cystinuria. This disorder runs in families and affects both men and women.

Struvite stonesare 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 stonesare more common in men than in women. They can occur with gout or chemotherapy[22].

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.

Fig.2: Different types of kidney stones



SYMPTOMS

- 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).
- 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[23]

Causes

- Low fluid intake
- High dietary intake of animal protein
- Sodium
- High fructose corn syrup
- Oxalate
- Grapefruit juice
- Apple juice
- Cola drinks[24]

Medications Increasing the Tendency for Stone Formation

- Acetazolamide
- Allopurinol
- Calcium carbonate
- Chemotherapy
- Sulfonamides
- Triamterene
- Vitamine C
- Vitamine D, steroids[25]

Medical management of kidney stones

There are a number of practices for treatment of urinary calculi, including surgery, and endoscopic procedures such as ureterscopy percutaneous nephlithotomy and extracorporeal shock wave lithotripsy. Medical management of urolithiasis is still a challenge for modern medical practice. Doctors can usually diagnose kidney stones by asking about symptoms and examining patient. Further tests may be done to confirm the diagnosis and to reveal the size, location and type of stone.

(i) **Blood tests**

These are done to identify excess amounts of certain chemicals related to the formation of stones and to check the presence of infection by blood cell counts.

(ii) **Urine analysis**

It helps to look for signs of infection and estimation of values of various contributing factors viz. oxalates, calcium, cystine, citrates, magnesium, phosphates, etc.

(iii) Taking an X-ray image

Stones that contain calcium are usually seen as white spots on X-ray images[26].

(iv) **An intravenous urogram (IVU)**

This involves an injection of a special dye that shows up the whole urinary system on X-ray images, revealing stones that can't usually be seen. Traditional intravenous pyelography is no longer the primary method of investigation in patients with renal colic[27].

(v) Abdominal Ultrasonography

Abdominal ultrasonography has limited use in the diagnosis and management of urolithiasis. Although ultrasonography is readily available, quickly performed and sensitive to renal calculi, it is virtually blind to ureteral stones (sensitivity: 19 percent), which are far more likely to be symptomatic than renal calculi[28].

(vi) Plain Film Radiography

Less radiopaque calculi, such as pure uric acid stones and stones composed mainly of cystine or magnesium ammonium phosphate, may be difficult, if not impossible, to detect on plain-film radiographs. Although 90 percent of urinary calculi have historically been considered to be radiopaque, the sensitivity and specificity of KUB radiography alone remain poor (sensitivity: 45 to 59 %; specificity: 71 to 77 %)[29].

(vii) Non-contrast helical computerized tomography

It produces pictures from a series of X-ray images taken at different angles - it is sometimes used to diagnose kidney stones and is thought to be the most accurate diagnostic test. It has become the first-line investigation in a number of centers. This imaging modality is fast and accurate and it readily identifies all stone types in all locations. Its sensitivity (95 to 100 percent) and specificity (94 to 96 percent) suggest that it may definitively exclude stones in patients with abdominal pain[30].

(viii) Shock wave lithotripsy

Shock wave lithotripsy is an external source to the patient that propagates through the body before being focused on kidney stone waves that cause stone fragmentation directly by producing mechanical stresses or indirectly by the collapse of cavitation bubbles. This is the most common treatment for urolithiasis, which can have slightly side effects[31].

(ix) **Extracorporeal Shockwave Lithotripsy (ESWL)**

ESWL is a non-invasive procedure which uses shock waves to fragment calculi. This proficiency is the most widely used method for dealing renal and ureteral stones. However, intervention success rates depend on stone composition, size, properties and location of the stone as well as the orchestration type and frequency of shock[32].

Some oral medicinal drugs have positive effects, they are not effective in all patients, but citrate is one of the majority widely used medical therapies for preventing urinary stone disease. The medical treatment of urolithiasis is aimed at assisting the patient from further

growth of existing stones and development of new stones, thus decreasing morbidity and the need for surgical intervention hence, under these circumstances medical treatment[33].

(x) Available Herbal Formulations for Urolithiasis

- Cystone
- Calcury
- Chandraprabhabati
- Trinapanchamool
- Rencare Capsule
- Patherina tablet
- BerPattharBhasma
- ChanderPrabha vati[34]

2. AIM AND OBJECTIVE

AIM

- The aim of the present study is to evaluate the anti-urolithiatic activity of various extracts of whole plants of *Justicia tranquebariensis* by *in vitro* and *in vivo* methods.

OBJECTIVES

- Collection and authentication of the plant *Justicia tranquebariensis*.
- Successive extraction of whole plant of *Justicia tranquebariensis* by hot percolation method using soxhlet apparatus.
- Evaluation of *in-vitro* anti-urolithiatic activity of whole plant of *Justicia tranquebariensis* by turbidity method and titrimetry method.
- To evaluate the anti urolithiatic activity of effective extract of *Justicia tranquebariensis* in ethylene glycol induced urolithias in male wistar rats.

3. REVIEW OF LITERATURE

1. R.Krishnamoorthi and V.RathaBai. (2015) investigated the possible phytochemical components from the solvents such as Hexane, ethyl acetate and Ethanolic extract of *Justicia tranquebariensis*. Among the phytochemical screening of these extracts ethyl acetate extract showed that the whole plant was rich in Carbohydrate, Tannin, Flavonoids, Quinones, Cardiac glycosides, Phenols, Seroids and Coumarins[35].

2. Akilandeswari, S.Manimaran*et al*; (2001) reported that phytochemical studies of leaf of herbs *Justicia tranquebariensis*. (Acanthaceae) carried out in the presence of Phytosterols, Flavonoids, Glycosides and absence of triterphenoids, alkaloids, saponins, tannins[36].

3. B.Saritha&P.Brindha. (2013) *Justicia tranqueibariensis*L.'belonging to the family acanthaceae on of the source of sivanarvembu is reviewed from ethnopharmacognostic. In Tamil it is called as "Tavashoomoorunghie" or "poonakapoondo". The present paper deals with the literature available on the ethno botanical pharmacognostic, phytochemical and pharmacological studies on *Justicia tranquebariensis* L. The review can help in detecting the selected drug from other sources of 'Sivanarvembu' and will also scientifically help in justifying its usage as 'Sivanarvembu"[37].

4. V.Velpandian *et al*; (2014) investigated 40 asthmatic patients were enrolled for the clinical trial. The patients were selected according to the patient subjective assessment scale, objective parameters and WHO INA guideline. The patients with severe disease such as AIDS, Malignanacy, TB, Renal and CVS disorders were excluded from the study, this open labeled study was conducted at Government Siddha Medical college hospital, Chennai-106. All the patients were administered 30ml leaf juice of *Justicia tranquebariensis* for the period of 3

months. Clinical trial usually focuses on asthma control as measured by pulmonary function test (FEV1, FVC, PEFR, Breath holding time FVR), symptom scores and medication requirement. The study infers improvement on subjective and objective parameters of bronchial asthma[38].

5. Radhika J *et al*; (2013) evaluated the cardioprotective role of *Justicia tranquebariebnsis* Linn. Leaf extract on isoproterenol induced myocardial infarction in Wistar albino rats. A rise in the levels of LDL, VLDL with significant decrease in the level of HDL was also observed in the serum of isoproterenol intoxicant rats. Significant increase in the level of myocardial marker enzymes (CK,LDH,ALT and AST) in serum was noted. The LDH & CK levels were low in heart tissue. Oral administration aqueous leaf extract of *Justicia trannquebariensis* Linn. (100 and 200mg/kg) to isoproterenol induced rats daily for a period of 28 days proved the role of the aqueous extract of *Justicia tranquebariensis*[39].

6. Shabana Begum *et al*; (2011) have been studied the protective and curative effect of *Justicia tranquebariensis* leaf extract using acetaminophen-induced liver injury in mice. The leaf extract at dosage of 500 and 1000mg/kg exhibited significant protective effect against acetaminophen induced hepatotoxicity. Level of serum markers such as aspartate amino transferase (AST), Alanine amino transferase (ALT), alkaline phophatase (ALP), and total bilirubin (TB) were significantly increased in acetaminophen treated mice. The result of the study confirmed the protective and curative effect of the aqueous leaf extract of *Justicia tranquebariensis*[40].

7. Begum, M.S *et al*; (2010) carried out antipyretic effect of aqueous extract of the leaves of *Justicia tranquebariensis* (Acanthaceae) at two doses. Subcutaneous injection of sterilized brewer's yeast suspended in 0.05 percent in saline at the dose of 10mg/kg body weight in albino

mice leads to pyrexia. The results concluded that the animals treated with 500 and 1000mg/kg doses of *Justicia tranquebariensis* exhibited significant antipyretic activity[41].

8. Jose A *et al*; (2009) evaluated that water and alcoholic extract of aerial parts of *Justicia tranquebariensis* (Acanthaceae) were subjected to various phytochemical analysis to identify carbohydrates, phytosterols, tannins and lignin. Both the extracts were investigated for diuretic activity. The result of the study confirmed the diuretic activity of *Justicia tranquebariensis*[42].

4. PLANT PROFILE

Botanical name: *Justicia tranquebariensis*

Family: Acanthaceae

Parts used: Whole plant

Fig.3: Whole plant of *Justicia tranquebariensis* (L)

Taxonomic Classification

Kingdom : Plantae

Division :Magnoliophyta

Class :Magnoliopsida

Order :Scrophulariales

Family :Acanthaceae

Subfamily :Acanthoideae

Genus :*Justicia*

Species :*tranquebariensis*[43]

Regional Names

Sanskrit : Pindi

Tamil : Sivanarvembu, tavashoomoorunghie

Oriya : Pindi

Telugu : Pindikonda, Chikerachettu, Kondapindi,

Redamandalam

Kannada : Shiva naaru balli, Kaddiyarakina, Kaddiyarakina gida[44,45]

Geographical Distribution

Deccan, Mysore, Karnataka, Southwards and also in all districts of Peninsular India and in Srilanka**.**

Siddha Uses

Leaf is used as expectorant, in Cold, Cough and nasal disorders[46].

Phytochemical Constituents

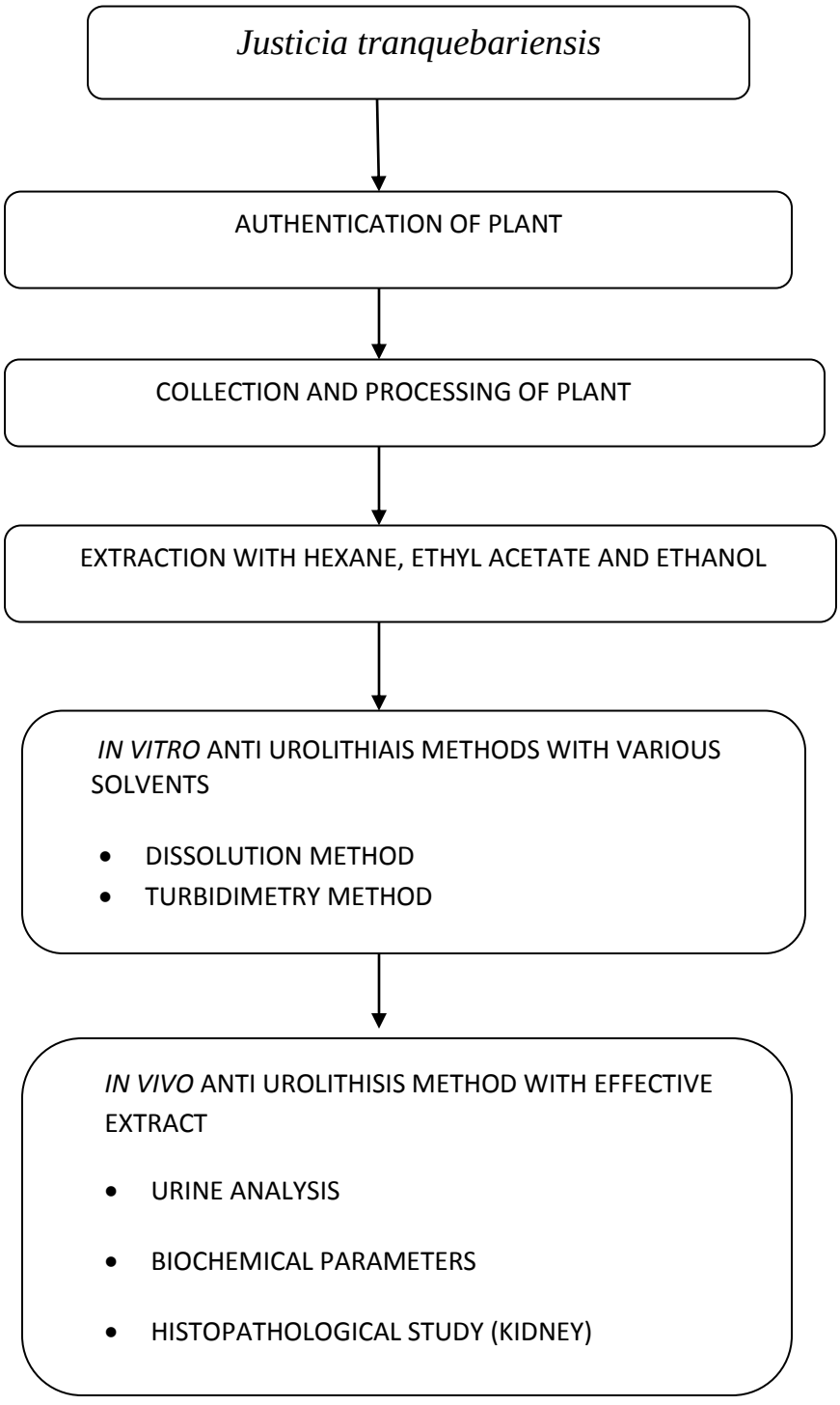
Phytochemical studies of leaf of the plant of *Justicia tranquebariensis*revealed the presence of phytosterols, flavonoids, Glycosides and absence of triterpenoids, alkaloids, saponins and tannins[36].

From Aerial Parts of *Justicia tranquebariensis*L.Lignans such as aryltetralin were isolated and characterized as (-)-beta-Cubebin, (+) –Lariciresinol, (+) –isolariciresinol, (+) –Lyoniresinol and (+) – MedioresinolLariciresinol and Isolariciresinol were proven to be anti inflammatory, antinociceptive, anti – ulcerogenic, antimicrobial , cytotoxic and antioxidant activites. Cubebin possess anti inflammatory activity. Lyoniresinol reveals antioxidative and also antimutagenetic activity. The alcoholic extract of the aerial part of *justicia tranquebarensis* yielded Phytosterols, brassicasterol, Campesterol, 7, 22 – ergostadienol, stigmasterol, sitosterol, spinasterol, 28-isofucosterol and betasitosterol-3-Oglucoside.

Medicinal Uses

Juices of leaves act as a cooling agent and aperients and also given to children in Small pox. Crushed leaves applied to contusions[47]. Paste made of the leaves applied externally on the swelling to reduce the pain. Root paste applied for tooth ache[48].Leaf juice, about15-20 ml, is administered orally for every one hour up tohalf of the day and keeping of leaf paste externally on the sight of snake bite work as an antidote for Cobrabite[49]. Leaf juice is given orally to treat jaundice and leaf paste is applied over affected area to treat skin diseases[50-52].

5. PLAN OF WORK



6. MATERIALS AND METHODS

Plant collection and identification

The whole plant was collected from Rapoosal, Pudukkottai District, Tamilnadu (INDIA) in the month of September 2018. It was authenticated by Dr. K N Sunil kumar, Research officer and Head Of the Department Pharmacognosy, Sidda Central Research Institute, Arumbakkam, Chennai.

Preparation of plant extracts

The powdered plant material (50g) was extracted by hot continuous soxhlet extraction method. The plant material was extracted with Hexane (500ml), Ethyl acetate (500ml) and Ethanol (99.9% v/v) (500ml), for four days in a soxhlet apparatus.

It is a process of continuous extraction method in which the solvent can be circulated through the extractor for several times. The vapours 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 Hexane, Ethyl acetate and Ethanol 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^{o}C), (35-40^{o}C), (40-45°C), (20-30^{o}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.

Drugs and chemicals

Hexane, Ethyl acetate, Ethanol, Cystone, Ethylene glycol.

PHYTOCHEMICAL ANALYSIS[53-61]

Test for Carbohydrates

To 2ml of plant extract, 1ml of Molisch's reagent and few drops of concentrated sulphuric acid were added. Presence of purple or reddish color indicates the presence of carbohydrates .

Test for Tannins

To 1ml of plant extract, 2ml of 5% ferric chloride was added. Formation of dark blue or greenish black indicates the presence of tannins .

Test for Saponins

To 2ml of plant extract, 2ml of distilled water was added and shaken in a graduated cylinder for 15minutes lengthwise. Formation of 1cm layer of foam indicates the presence of saponins.

Test for Flavonoids

To 2ml of plant extract, 1ml of 2N sodium hydroxide was added. Presence of yellow color indicates the presence of flavonoids.

Test for Alkaloids

To 2ml of plant extract, 2ml of concentrated hydrochloric acid was added. Then few drops of Mayer's reagent were added. Presence of green color or white precipitate indicates the presence of alkaloids.

Test for Quinones

To 1ml of extract, 1ml of concentrated sulphuric acid was added. Formation of red color indicates presence of quinones.

Test for Glycosides

To 2ml of plant extract, 3ml of choloroform and 10% ammonia solution was added. Formation of pink color indicates presence of glycosides.

Test for Cardiac Glycosides

To 0.5ml of extract, 2ml of glacial acetic acid and few drops of 5% ferric chloride were added. This was under layered with 1 ml of concentrated sulphuric acid. Formation of brown ring at the interface indicates presence of cardiac glycosides.

Test for Terpenoids

To 0.5ml of extract, 2ml of chloroform was added and concentrated sulphuric acid was added carefully. Formation of red brown color at the interface indicates presence of terpenoids.

Test for Phenols

To 1ml of the extract, 2ml of distilled water followed by few drops of 10% ferric chloride was added. Formation of blue or green color indicates presence of phenols .

Test for Coumarins

To 1 ml of extract, 1ml of 10% NaOH was added. Formation of yellow color indicates presence of coumarins.

Phlobatannins

To 1ml of plant extract few drops of 2% HCL was added appearance of red color precipitate indicates the presence of phlobatannins.

Steroids and Phytosteroids

To 1ml of plant extract equal volume of chloroform is added and subjected with few drops of concentrated sulphuric acid appearance of brown ring indicates the presence of steroids and appearance of bluish brown ring indicates the presence of phytosteroids.

Anthraquinones

To 1ml of plant extract few drops of 10% ammonia solution was added, appearance pink color precipitate indicates the presence of anthraquinones.

IN-VITRO ANTI-UROLITHIATIC ACTIVITY BY TURBIDITY METHOD AND TITRIMETRY METHOD

TURBIDITY METHOD[62-64]

Principle

In vitro anti-urolithiatic activity of *Justicia tranquebariensisa* whole plant extract were tested in terms of inhibition of calcium oxalate formation by the extracts in the presence (standard drugs and extract) and absence of inhibitors. The precipitation of calcium oxalate at 37^{o}C and pH 6.8 has been studied by the measurement of turbidity by UV/Vis spectrophotometer at 620nm. It was employed to measure the turbidity caused due to formation of calcium oxalate. To evaluate calcium oxalate inhibition of plant extracts by absorptions were noted and in microscopical study the comparison of un-controlled growth of the stone nucleus for the comparison of growth in the presence of the standard drugs and plant extracts were also observed.

Materials requirements

- Calcium chloride dihydrate
- Sodium oxalate
- Tris buffer
- Cystone 750mg
- Hexane, ethyl acetate and ethanolic Plant extracts.

Procedure

Study without inhibitor

Volume of 1.0 ml of 0.025M calcium chloride dihydrate and 2ml of Tris-buffer (pH7.4) were added in a test tube. Then 1.0ml of 0.025M sodium oxalate was added. After mixing of above solution immediately due to the formation of turbidity and then up to the period of 10 minutes to measure the turbidity of solution by UV/Vis spectrophotometer at 620nm. This control experiment was done in three replications.

Study with inhibitor

In this experiment, 1ml of 0.025M calcium chloride dihydrate, 2ml Tris-buffer and 1ml (10 mg/ml solution) of Hexane, ethyl acetate and ethanolic plant extracts were added in a four sets of test tubes. Two more test tubes were added 1ml of 0.025M calcium chloride dihydrate, 2ml Tris-buffer and poly herbal formulation, Cystone. Then 1ml of 0.025M sodium oxalate was added to each test tube and then up to the period of 10 minutes to measure the change in turbidity of the solution by UV/Vis spectrophotometer at 620nm. Each procedure was done in three times.

Inhibition in stone nucleus formation was calculated by graphical method using the following formula:

$$\textbf{Percentage inhibition} = \{1-[Si/Sc]\} \times 100$$

Where;

Si - slope of graph in the presence of inhibitors (drugs/plant extracts)

Sc - slope of without inhibitors (control).

Microscopic study

Light microscopy of the crystals formed in the solution with and without was also done. Photographs of calcium oxalate were taken using the objective of 40X.

TITRIMETRY METHOD[65-68]

Principle

The Titrimetric method is one of dissolution method, the artificial preparation of calcium oxalate crystal was taken in the egg semipermeable membranes act as control (without inhibitor) and added different plant extracts and standard Cystone (with inhibitor). Then it was immersed in the 0.1M Tris buffer solution and incubated at 37^{o}C for 2hours. After 2hours removal of content of semi permeable membranes and add 2ml of 1N sulphuric acid titrated against 0.9494N potassium permanganate till a light pink colour end point was obtained. The amount of remaining undissloved calcium oxalate is subtracted from the total quantity used in the experiment in the beginning to know the total quantity of dissolved calcium oxalate by various extracts.

Material required

- Calcium chloride dihydrate
- Sodium oxalate
- Tris buffer
- Egg semipermeable membrane
- Cystone 750mg
- Hexane, ethyl acetate and ethanolic Plant extracts.
- Sulphuric acid

Procedure

The dissolution method involved the three steps.

They are,

- Preparation of experimental kidney stones (calcium oxalate stones) by homogenous precipitation.
- Preparation of semi-permeable membrane from eggs.
- Estimation of calcium oxalate by Titrimetry.

Step -1: Preparation of experimental kidney stones (calcium oxalate stones) by homogenous precipitation

1.34gm of sodium oxalate was dissolved in 100 ml of 2N H_2SO_4 and1.47gm of calcium chloride dihydrate was dissolved in 100ml distilled water. Both were mixed equally in a beaker to precipitate out calcium oxalate. The precipitate freed from traces of H_2SO_4 by ammonia solution. Washed the precipitates with distilled water and dried at 60^oC for 4hours.

Step -2:Preparation of semi-permeable membrane from eggs

The semi - permeable membrane of eggs lies in between the outer calcified shell and the inner contents like albumin &yolk. Apex of eggs was punctured by a glass rod in order to

Squeeze out the entire content. Empty eggs were washed thoroughly with distilled water and placed in a beaker consisting 2M Hcl for an overnight, which caused complete decalcification. Further, washed with distilled water, placed it in ammonia solution for neutralization of acid traces in the moistened condition for a while & rinsed it with distilled

Water. Stored in refrigerator at a pH of 7- 7.4.

Step -3: Estimation of calcium oxalate by Titrimetry

The dissolution percentage of calcium oxalate was calculated by taking exactly 1 mg of calcium oxalate and 10mg of different plant extracts, packed it together egg semipermeable membrane. This was allowed to suspend in a conical flask containing 100ml of 0.1M Tris buffer. The1 mg of calcium oxalate in egg semipermeable membrane act as the control. The1 mg of calcium oxalate and 10 mg of Cystone (standard) and 10mg of Hexane, ethyl acetate and ethanolic Plant extracts in egg semipermeable membrane. All the conical flask containing semipermeable membrane were kept in an incubator to 37^{o}Cfor 2hours.Remove the contents of semipermeable membrane in to separate test tube, add 2ml of 1Nsulphuric acid to each test tube and titrated with 0.9494N KMnO4 till a light pink colour end point obtained. The amount of remaining undissloved calcium oxalate is subtracted from the total quantity used in the experiment in the beginning to know the total quantity of dissolved calcium oxalate by various extracts.Each ml of 0.9494 N $KMnO_4$ equivalents to 0.1898mg of Calcium oxalate.

The percentage dissolution calculated follows

Dissolved calcium oxalate = (Total quantity used in the Experiment in the beginning)

- (Undissloved calcium oxalate)

Percentage dissolution=Dissolved calcium oxalate X 100

IN-VIVO STUDIES

MAINTENANCE OF ANIMALS

Experimental Animal

The present study was conducted as per CPCSEA/IAEC approval no: 5/AEL/IAEC/MMC, Date:12.9.2018. The Male Wistar albino rats (150-200g) used for this study were produced from Animal Experimental Laboratory, 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.

ACUTE TOXICITY STUDY[69]

The acute toxicity study has been carried out already **.**The non-toxic nature of the ethanol extract is evident from the acute oral toxicity conducted as per OECD guidelines (Organisation of Economic Co-operation and development) 423(Acute Toxic Class Method). The normal behaviour of the test animals during a period of 14 days suggests the non-toxic nature of the fore said extracts. Hence waterhyacinth could be safe up to the dose of 2000 mg/kg body weight of the animal. Further studies are warranted for determining chronic toxic symptoms.

***IN-VIVO* ANTI-UROLITHIASIS EVALUATION**

The antiurolithiatic activity of *Justicia tranquebariensis* 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.

A) Ethylene Glycol Induced Urolithiasis Model[70]

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

Table 1: *In vivo* anti-urolithiasis Experimental design

Group	Treatment	No. of animals
I	Regular diet and water	**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 (15-28th day)	**6**
IV	Ethylene Glycol (0.75% v/v) for 28 days + Effective extract 200mg/kg (1-28th day)	**6**
V	Ethylene Glycol (0.75% v/v) for 28 days + Effective extract 400mg/kg (1-28th day)	**6**

B) ASSESSMENT OF ANTIUROLITHIATIC ACTIVITY

a) URINE ANALYSIS

On 28th day 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^{o}C. Urine was analyzed for urine volume, pH, calcium, phosphate, oxalate and magnesium.

i) Estimation of Calcium in Urine (Bio Chain)[71]

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 hyper vitaminosis, while decreased levels are observed in hyperparathyroidism, 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 pre treatment. 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 4ºC 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 labelled tubes.

2. Add 1000ml working reagent and vortex to mix. Incubate 3min. Transfer to cuvette 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

Calcium concentration of the sample=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).

ii) Estimation of Magnesium in Urine[72]

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**
L_1: Buffer reagent	12.5ml	37.5ml
L_2: 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 at2-8^{o}C for at least one month keep tightly closed.

Sample material

Urine: 24hr collected urine should be acidified to pH of 2-3 by the addition ofapprox.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 labelled as Blank (B), Standard (S) and Test (T).

Addition sequence	Blank (ml)	Standard (ml)	Test (ml)
Buffer reagent (L_1)	0.5	0.5	0.5
Colour reagent (L_2)	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

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

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

iii) Estimation of Phosphorus in Urine[71]

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 are 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 $\longrightarrow$ Phosphomolybdate complex

Contents	**75ml**	**2 x 75ml**
L_1: Acid Reagent	60ml	2 x 60ml
L_2: Molybdate Reagent	15ml	2 x 15ml
S: Phosphorus Standard (5mg/dl)	5ml	5ml

Storage/ stability

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

Working Reagent

Pour the contents of 1 bottle of L_2 (Molybdate Reagent) into 1 bottle of L_1 (AcidReagent). This working reagent is stable for at least 6 months when stored at 2-8ºC. 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 workingreagent 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 sequences	Blank (ml)	Standard (ml)	Test (ml)
Working reagent	1.0	1.0	1.0
Distilled water	0.01	-	-
Phosphorus Standard (S)	-	0.01	-
Sample	-	-	0.0

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 : 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

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

b) 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.

i) Estimation of Blood Urea Nitrogen[73]

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 condense 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 + Diacetylmonoxime ⟶ Red Coloured Complex

Contents

L_1: Urea Reagent

L_2: Acid Reagent

L_3: DAM Reagent

S: Urea Standard (40mg/dl)

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

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
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

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

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

Blood Urea Nitrogen = Urea in mg/ dl x 0.467

ii) Estimation of Uric Acid in Serum[73]

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.

Uric acid + H_2O ⟶ Allantoin + H_2O_2

H_2O_2 + 4 Aminoantipyrine + Phenolic Compound ⟶ (Peroxidase) **Red Quinoneimine dye + H_2O**

Contents: 25ml 75ml 2X75ml 2X150ml

L1: Buffer reagent 20ml 60ml 2X60ml 2X120ml

L2: Enzyme reagent 5ml 15ml 2X15ml 2X30ml

S: Uric acid Standard 5ml 5ml5ml5ml
(8mg/ dl)

Storage/ stability

All reagents are stable at 2-8^oC till the expiry mentioned on the label.

Working reagent

Pour the contents of 1 bottle of L_2 (Enzyme Reagent) into 1 bottle of L_1 (Bufferreagent). This working reagent is stable for at least 4 weeks when stored at 2-8^oc. 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

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	0.02	-	-
Phosphorus Standard (S)	-	0.02	-
Sample	-	-	0.02

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^{0}c/ room temp. Linearity: 20mg/dl

Incubation time : 5 min / 15 min Unit: mg/dl

Standard : 8mg/ dl

Calculation

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

iii) Estimation of Creatinine in Serum[73]

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

Creatinine + 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	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

Picric acid reagent (L_1)	2.0ml
Sample	0.2ml

Deproteinization of specimen

Pipette into a clean dry test tube

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

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

STATISTICAL ANALYSIS

Results were expressed as Mean ± Standard Error of Mean (SEM). $p<0.05$ was considered statistically significant. Data obtained was analysed by one-way ANOVA followed by Dunnett's multiple comparisons test using Graph pad prism version 7.

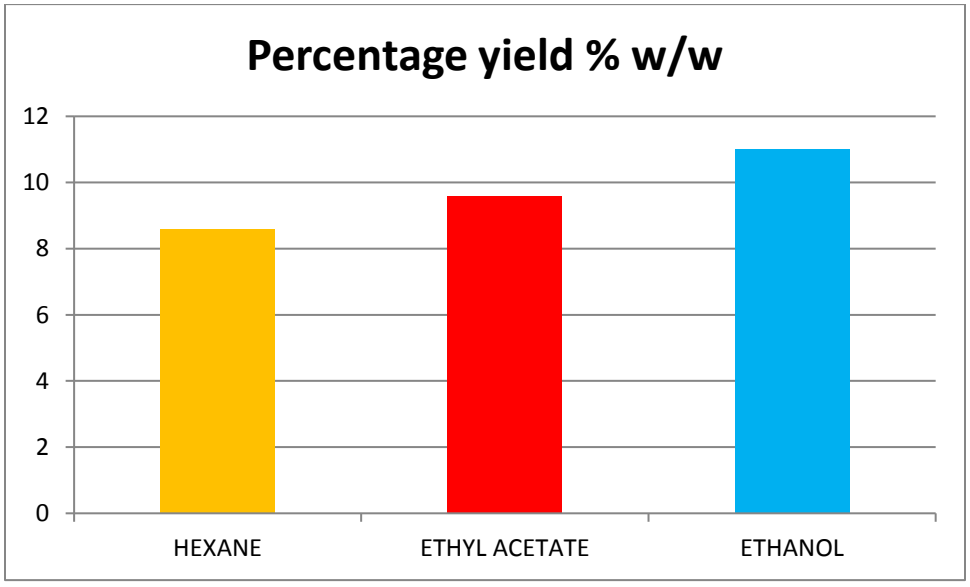
7. RESULTS

PERCENTAGE YIELD

Table 2: Percentage yield of the *Justicia tranquebariensis*Linn

S.no	Amount of whole plant (g)	Solvent used	Percentage yield (%w/w)
1.	50	Hexane	8.6
2.	50	Ethyl acetate	9.6
3.	50	Ethanol	11.0

Fig.4: percentage yield of *Justicia tranquebariensis*



PHYTOCHEMICAL ANALYSIS

Hexane, Ethyl acetate and Ethanolic extracts of whole plant of *Justicia tranquebariensis* was subjected to various chemical tests for detection of phyto constituents and results obtained are illustrated in Table 3 .

Table 3: Phytochemical analysis of Hexane, Ethyl acetate and Ethanolic extracts of whole plant of *Justicia tranquebariensis*

S.no	Phytochemical Test	Hexane extract	Ethyl acetate extract	Ethanolic extract
1.	Carbohydrates	-	-	-
2.	Tannins	-	-	-
3.	Saponins	-	-	+
4.	Flavonoids	-	+	+
5.	Alkaloids	+	+	+
6.	Quinones	-	-	+
7.	Glycosides	-	-	+
8.	Cardiac glycosides	-	-	+
9.	Terpenoids	-	-	-
10.	Phenols	-	-	-
11.	Coumarins	-	+	+
12.	Phlobatannins	-	-	-
13.	Steroids &phytosteroids	+	-	+
14.	Anthraquinone	-	-	-

NOTE: (+) Present (-) Absent

7.1 *IN-VITRO* ANTI-UROLITHIATIC ACTIVITY BY TURBIDITY METHOD AND TITRIMETRY METHOD

Turbidity method

Table 4: *In-vitro* anti-urolithiatic activity

s.no	Concentration (µg/ml)	% inhibition			
		Hexane extract	Ethyl acetate extract	Ethanolic extract	Cystone Standard
1.	200	21.34±0.23	24.12±0.32	32.22±0.14	34.65±0.16
2.	400	24.54±0.76	31.18±0.23	43.34±0.76	49.56±0.23
3.	600	36.43±0.24	38.67±0.83	50.38±0.50	53.45±0.34
4.	800	43.46±0.82	46.30±0.45	62.44±0.23	67.55±0.22
5.	1000	61.42±0.65	62.42±0.56	73.82±0.22	76.65±0.43
	IC_{50}(µg/ml)	**842.3**	**796**	**600.75**	**546.8**

Values are expressed as Mean±SEM (n=3)

From **table 4**, The ethanolic extract of *Justicia tranquebariensis* whole plant showed higher calcium oxalate crystallization inhibition (**73.82 %**) than the hexane (**61.42%**) and ethyl acetate (**62.42%**) extract of *Justicia tranquebariensis* whole plant for the turbidity method. While Cystone showed highest inhibition (**76.65%**) compared with plant extracts.

The IC_{50} value of ethanolic extract of *Justicia tranquebariensis* whole plant showed significant IC_{50} value (**600.75µg/ml**) than the hexane (**842.3µg/ml**) and ethyl acetate **(796µg/ml)** extract of *Justiciatranquebariensis* whole plant. The IC_{50}value of the Cystone were (**546.8 µg/ml**).

Fig.5: Effect of various extract of *Justicia tranquebariensis* on calcium oxalate crystallization inhibition

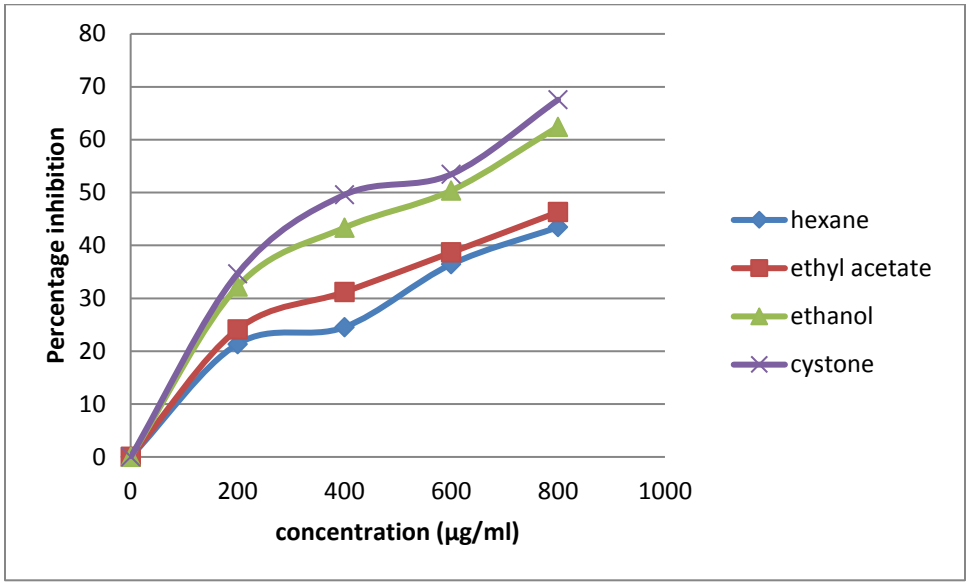


Fig.6: IC_{50} Value of Hexane, Ethyl acetate and Ethanolic extracts of *Justicia tranquebariensis* .

Fig.6.1: Hexane extract

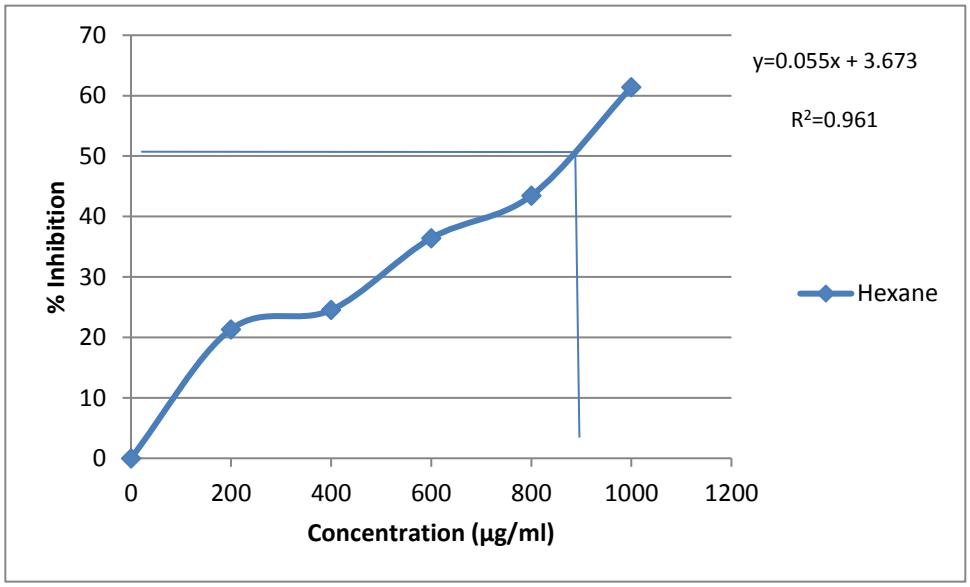


Fig.6.2 : Ethyl acetate extract

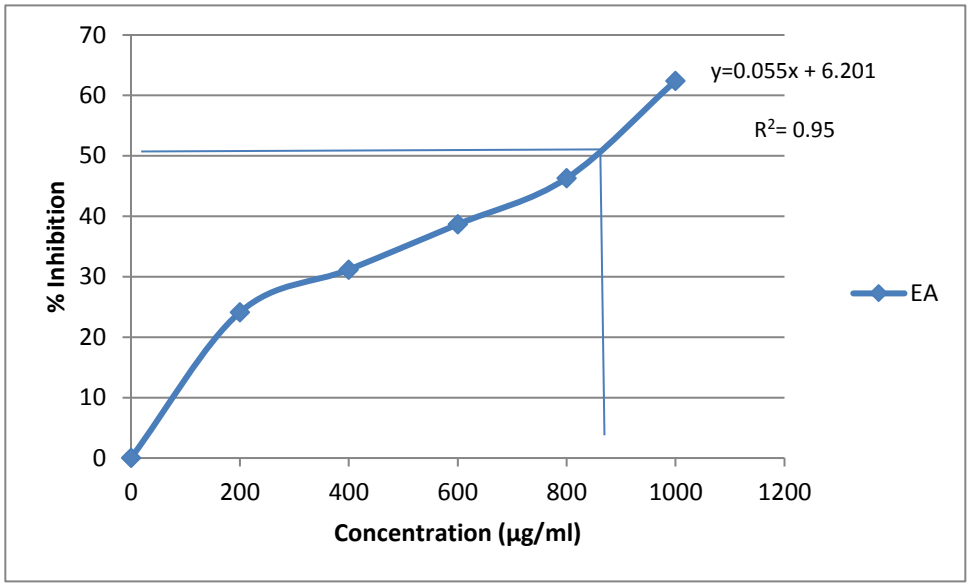


Fig.6.3: Ethanolic extract

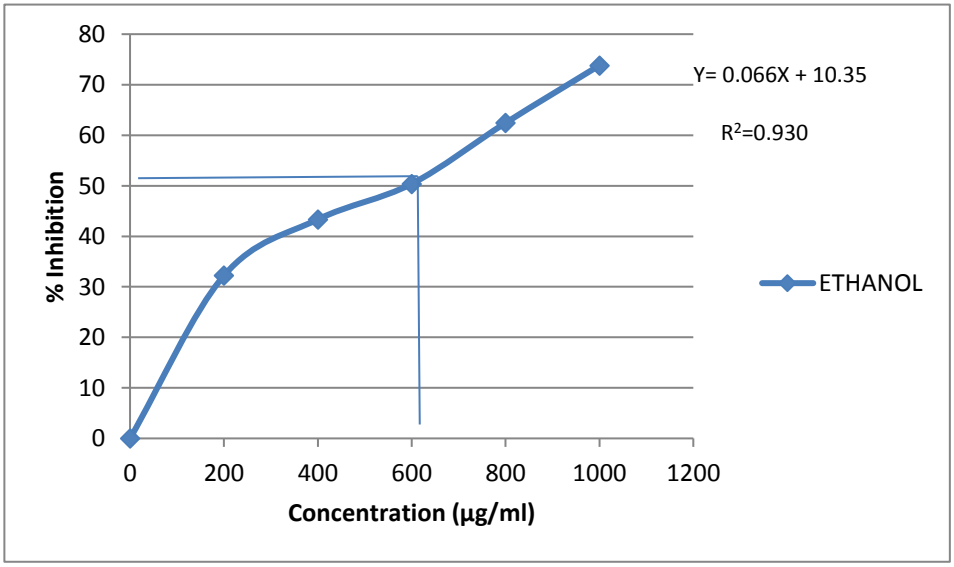


Fig.6.4: Cystone standard

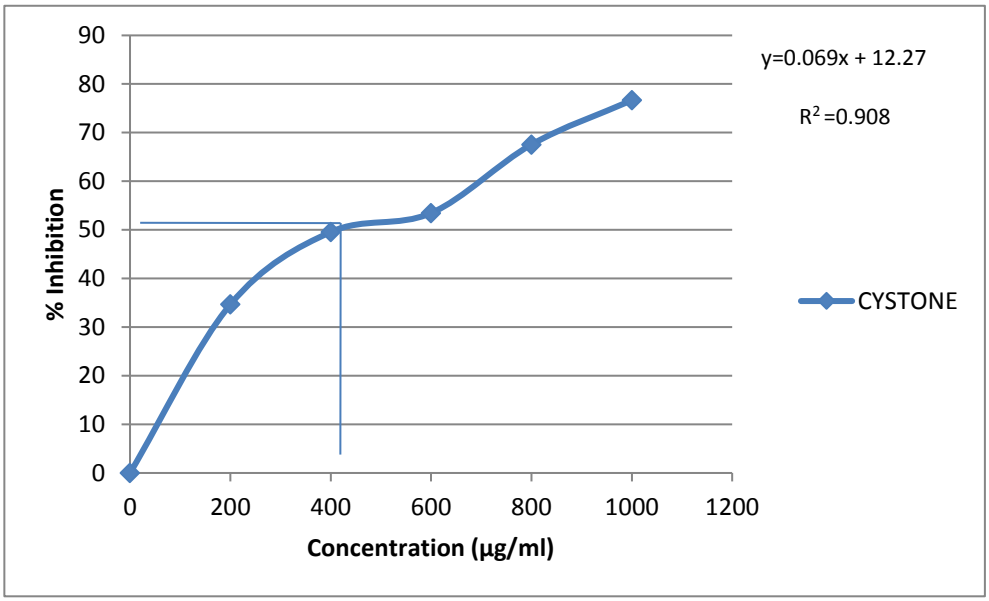
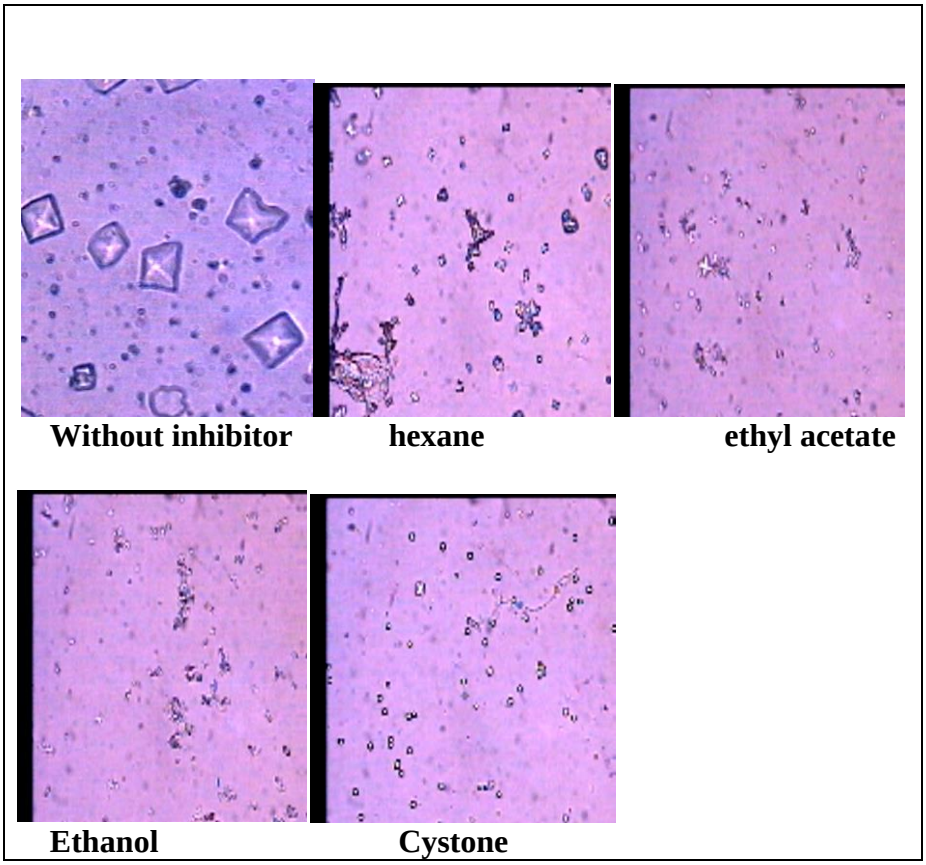


Fig.7: Formation of calcium oxalate nucleus and inhibition by plant extract and standard drug in turbidity method.



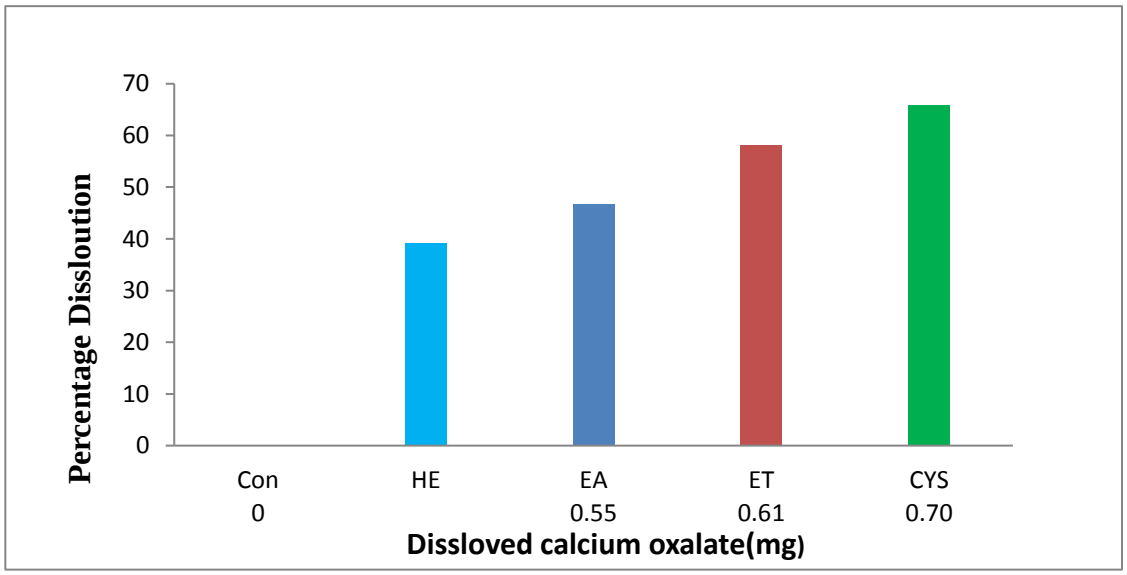
Titrimetry method

Table 5: The Percentage dissolution of calcium oxalate

S.no	Extracts	Dissolved calcium oxalate(mg)	% Dissolution
1	**Hexane extract**	0.392	39.2
2	**Ethyl acetate extract**	0.468	46.8
3	**Ethanolic extract**	0.582	58.2
4	**Cystone**	0.658	65.8

From **Table 5**, The ethanolic extract of *Justicia tranquebariensis* whole plant showed higher (**58.2%)** percentage dissolution of calcium oxalate than the hexane (**39.2%**) and ethyl acetate (**46.8%**) extract of *Justicia tranquebariensis* whole plant. While Cystone showed highest percentage dissolution (**65%**) of calcium oxalate by the Titrimetry method.

Figure.8: Calcium oxalate dissolved with different plants extracts (Hexane (HE), Ethyl acetate (EA), Ethanol (ET) and standard Cystone (CYS).



7.2 *IN-VIVO* ANTI-UROLITHIAITIC ACTIVITY

URINE ANALYSIS

i)Estimation of urine volume and pH

Table 6: Estimation of urine volume and pH

Parameters	Group-I	Group -II	Group-III	Group-IV	Group -V
URINE VOLUME	2.57±0.11	1.60±0.23$^{###}$	5.40±0.14***	3.35±0.54***	5.19±0.32***
pH	7.25±0.04	5.7±0.13$^{###}$	7.29±0.45***	6.30±0.24***	7.24±0.04***

Values are expressed as Mean ± SEM, n=6.

$^{*}P \leq 0.05$, $^{**}P \leq 0.01$ and $^{***}P \leq 0.001$ compared with disease control

From **Table 6**, It can be seen that Urolithiasis induced animals shows decreased in urine volume (Group II), whereas the treated groups (II, III, IV) shows increased in urine volume when compared with Group II rats.

In ethylene glycol induced rats pH was reduced when compared with normal control group. Treatment with cystone 750mg/kg and ethanolic extracts of *Justicia tranquebariensis* were found to increase the urine pH in a dose dependent manner.

Fig.9: Estimation of urine volume

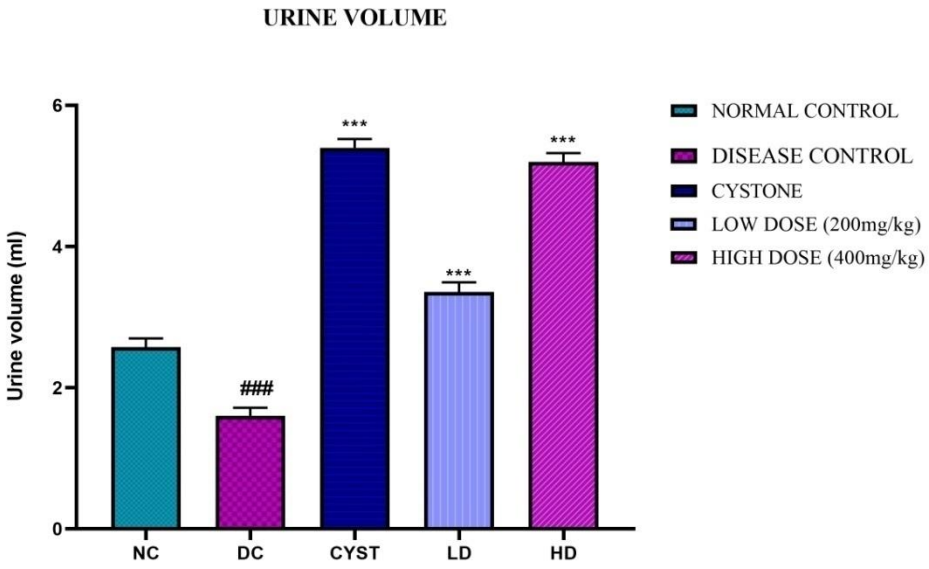
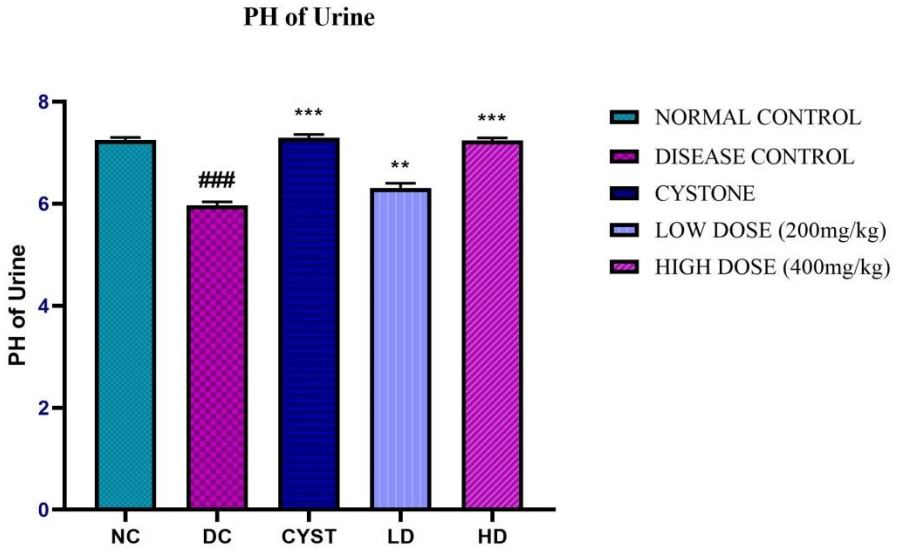


Fig.10: Estimation of urine pH



ii)Estimation of Calcium, Oxalate, phosphorus and magnesium in Urine

Table 7: Estimation of calcium, oxalate, phosphorus and magnesium in urine

Parameters	Group-I	Group-II	Group-III	Group-IV	Group-V
Calcium	9.54±0.10	13.56±0.08$^{###}$	10.24±0.11***	12.81±0.15**	10.36±0.169***
Oxalate	1.86±0.07	4.39±0.17$^{###}$	2.11±0.07***	3.8±0.12**	2.55±0.10***
Phosphorus	5.15±0.20	7.13±0.17$^{###}$	5.18±0.14***	6.33±0.14**	5.28±0.11***
magnesium	4.16±0.08	1.73±0.12$^{###}$	3.33±0.11***	2.11±0.09*	3.18±0.07***

Values are expressed as Mean ± SEM, n=6.

$^{*}P \leq 0.05$,$^{**}P \leq 0.01$ and$^{***}P \leq 0.001$ compared with disease control

From table 7, In disease control animals calcium, phosphorus and oxalate excretion were significantly increased, While the magnesium level decreased when compared with group I animals. When supplement with plant extract significantly lowered the elevated levels of calcium, phosphorus and oxalate when compared with group II animals, and restore the magnesium level when compared with normal animals.

Fig.11: estimation of calcium in urine

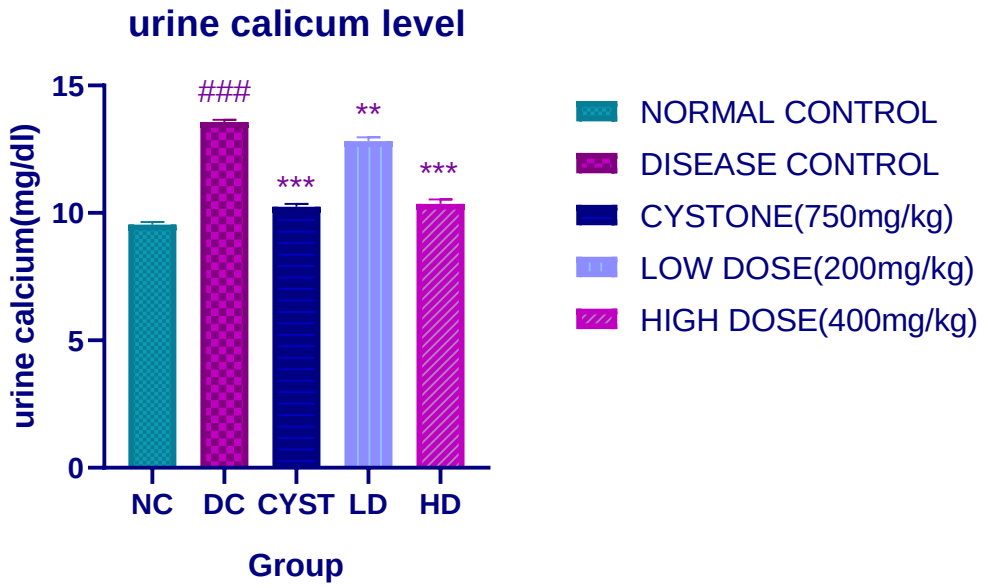


Fig.12: Estimation of oxalate in urine

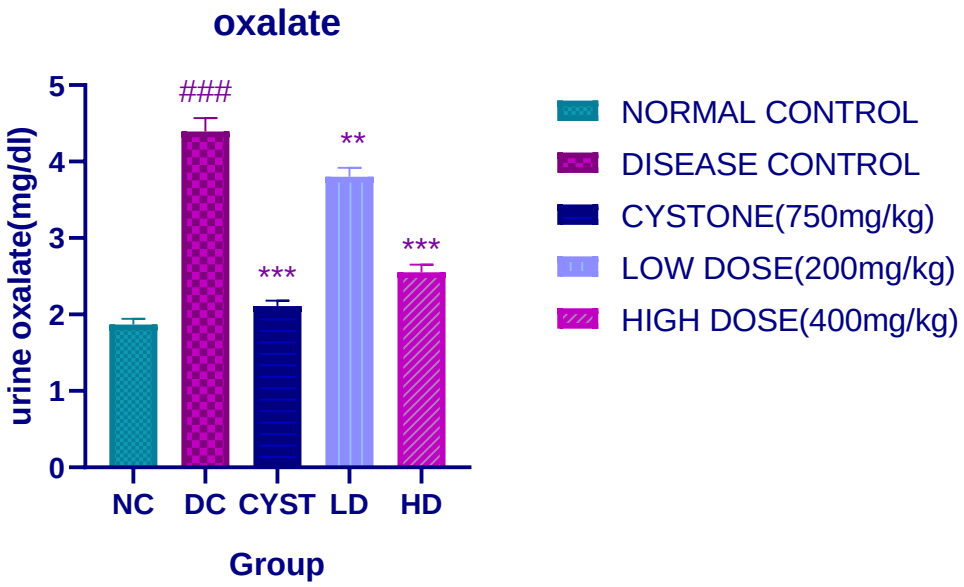


Fig.13: Estimation of phosphorus in urine

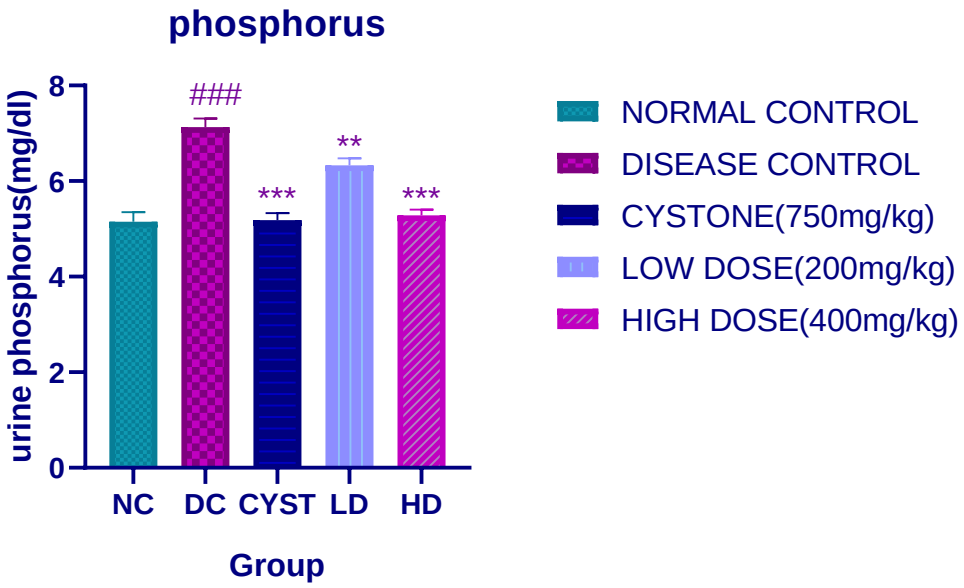
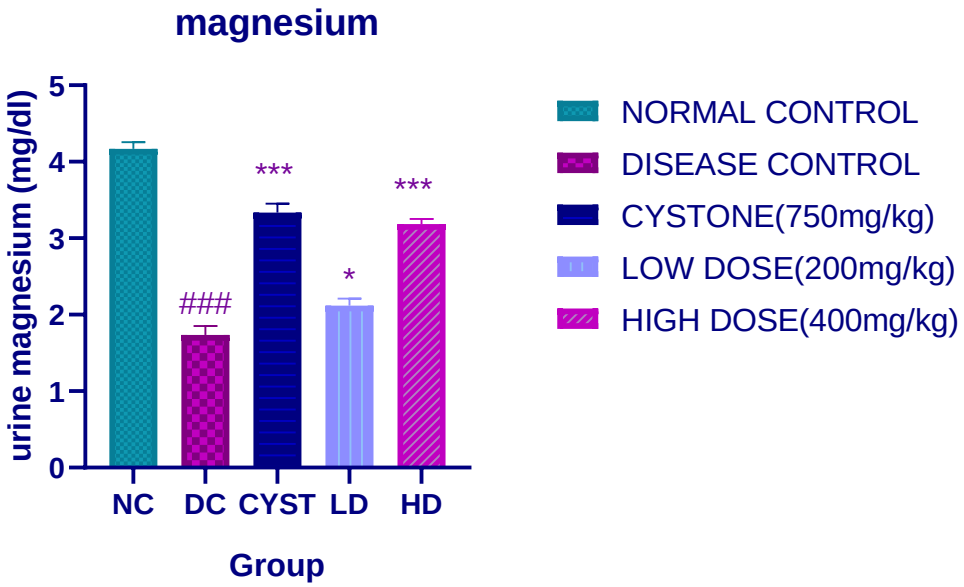


Fig.14: Estimation of magnesium in urine



Serum analysis

ii)Estimation of creatinine, BUN& uric acid in serum

table 7: Estimation of creatinine, BUN& uric acid in serum

parameters	Group-I	Group-II	Group-III	Group-IV	Group-V
BUN	16.88±0.25	33.99±0.26$^{###}$	21.4±0.41***	32.37±0.25**	25.31±0.16***
Ceatinine	2.65± 0.12	3.33±0.08$^{###}$	2.43±0.11***	2.91±0.07***	2.63±0.11***
Uric acid	2.7±0.05	4.43±0.09$^{###}$	2.95±0.07***	3.98±0.09**	3.21±0.08***

Values are expressed as Mean ± SEM, n=6.

$^{*}P \leq 0.05$,$^{**}P \leq 0.01$ and$^{***}P \leq 0.001$ compared with disease control

In calculi-induced rats (Group II), the elevated serum levels of creatinine, uric acid and Blood Urea Nitrogen (BUN). However, treatment with plant extract restored the serum levels of creatinine, uric acid and BUN. The ethanolic plant extract (Group IV and V) and Cystone (Group III) significantly ($p<0.05$) reduced the elevated serum uric acid level as compared to group II.

Fig.15: Estimation of BUN in serum

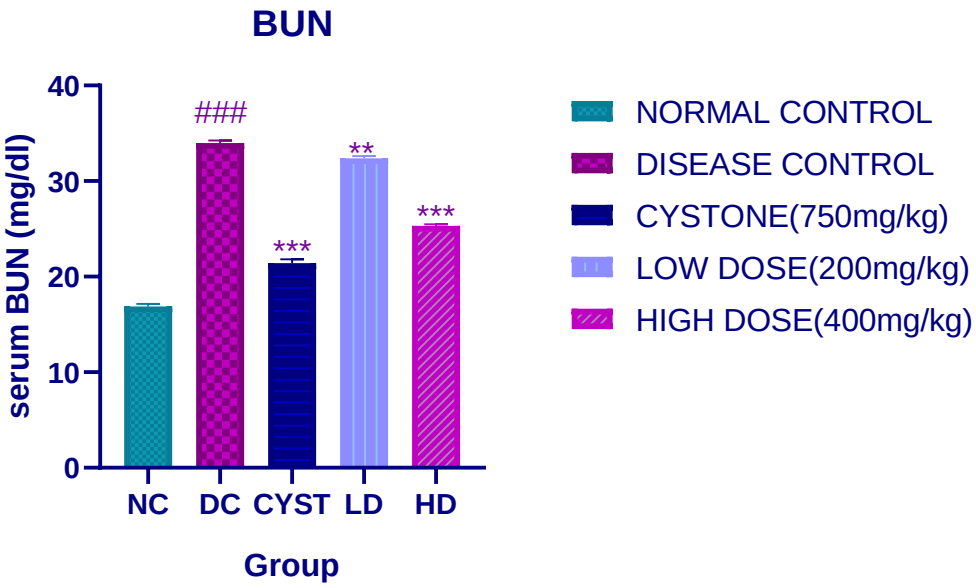


Fig.16: Estimation of creatinine in serum

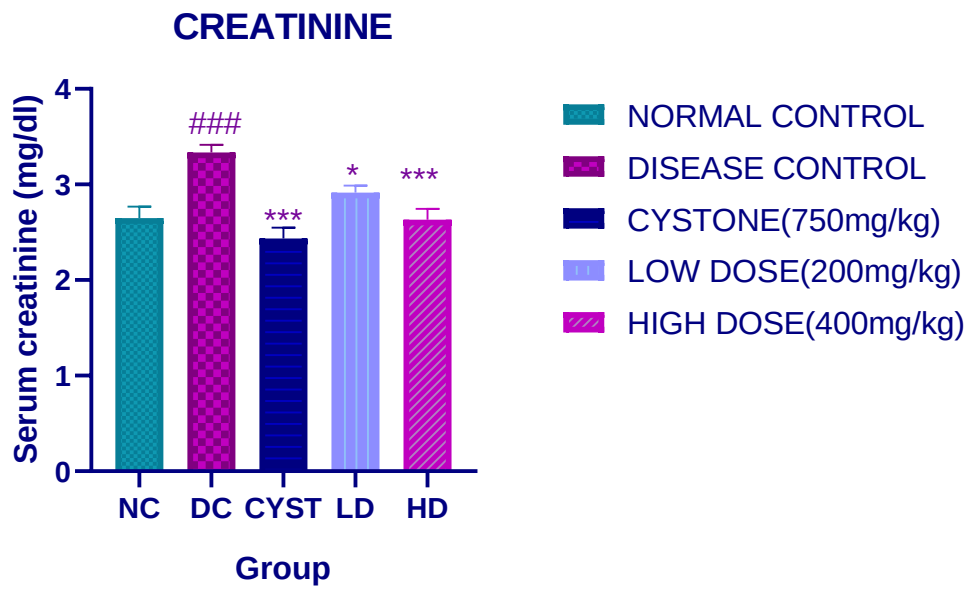


Fig.17: Estimation of uric acid in serum

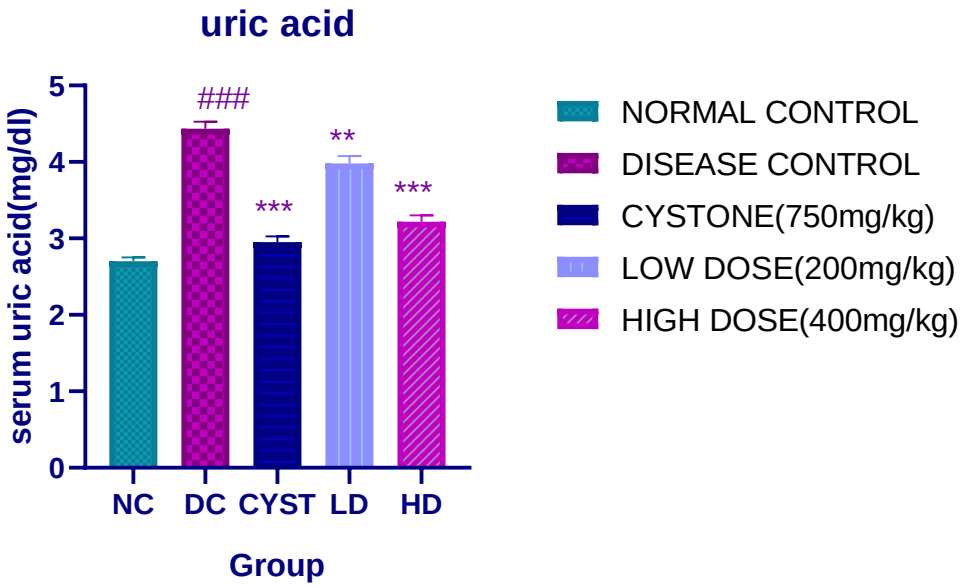
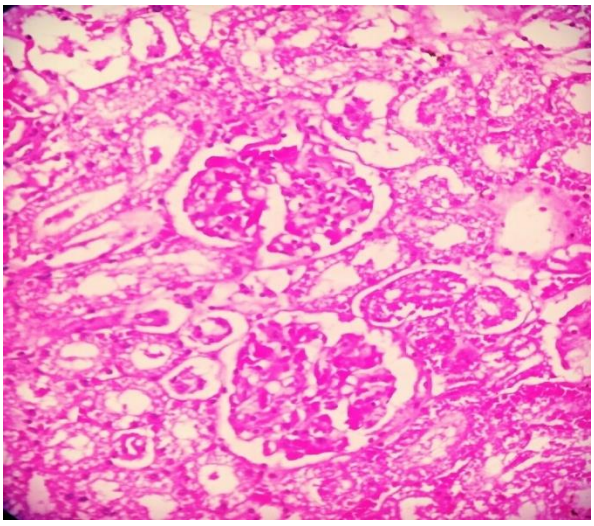
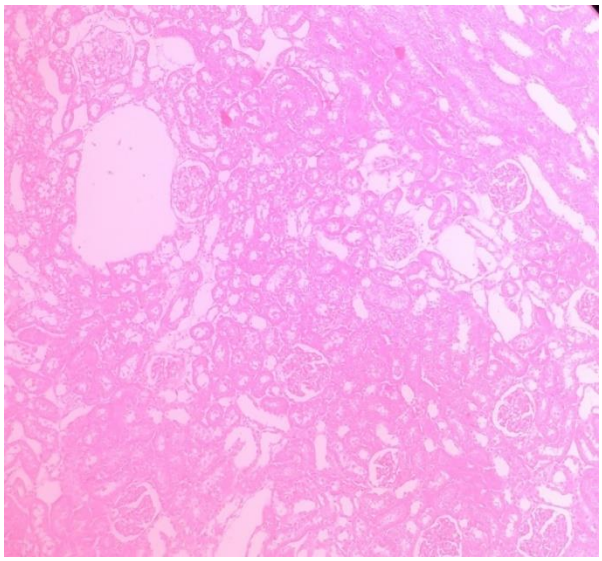


Fig 18 : Histopathological examination of rat kidneys

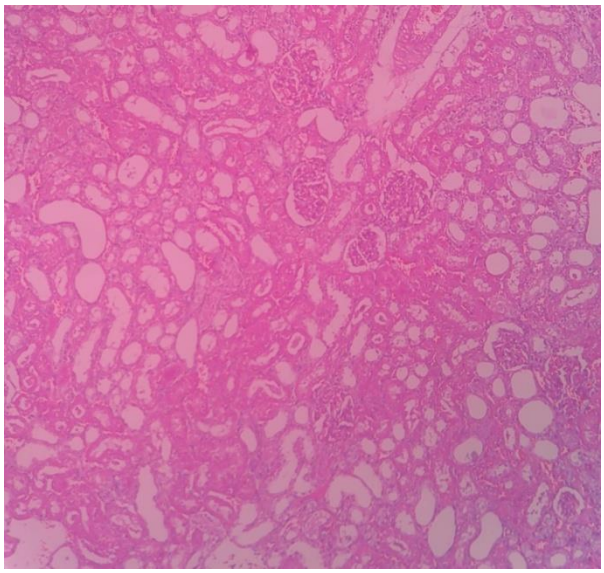
Normal control group



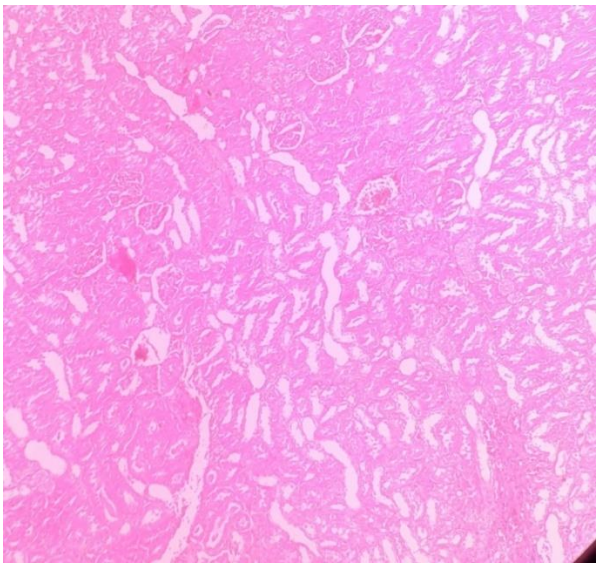
Disease control group



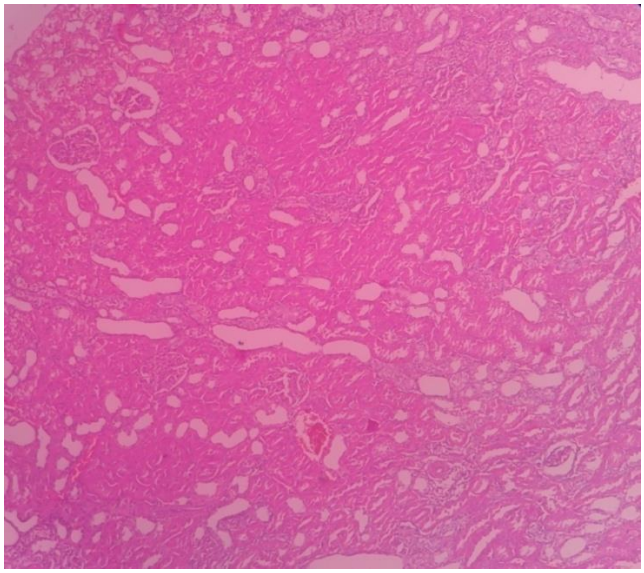
Standard treated group



Plant extract low dose (200mg/kg) treated group



Plant extract high dose(400mg/kg) treated group



From **fig.18,** in EG treated animal showed abnormal crystal deposition, more tubular dilation and large spaces. In treatment groups less crystal deposition, less dilation was seen.

8. DISCUSSION

Phytotherapy is a science-based medical practice and thus is distinguished from other, more traditional approaches, such as medicinal medical herbalism which relies on an empirical appreciation of medicinal herbs and which is often linked to traditional knowledge[74].

India, as in many less developed areas, phytotherapy is a common method of primary health care. *Justicia tranquebariensis* is small shrub, which is widely distributed in southern parts of India. Some species of the genus *Justicia* have been used in the traditional system of medicine for the treatment of various diseases.

In kidney, stone formation, calcium oxalate and calcium phosphates, or other chemicals in the urine form crystals on the inner surfaces of kidneys. This stage is called is initial mineral phase formation. Over a period of time crystals may combine to form a small, or mass called as stones and stage is referred as crystal growth.

Preliminary phyto-chemical analysis of hexane, ethyl acetate and ethanolic extract of whole plant of *Justicia tranquebariensis* revealed the presence of various components flavanoids, glycosides, quinines, caumarins, sopponins and steroids. Active phyto chemical such as flavanoids and sapponins are known to responsible for the diuretic activity.

The percentage inhibition of calcium oxalate crystallization of various extracts was calculated by using formula. when compared these three extracts, present study clearly indicates the ethanolic extract of *Justicia tranquebariensis* whole plant showed higher calcium oxalate crystallization inhibition (**73.82 %**) than the hexane (**61.42%**) and ethyl acetate (**62.42%**) extract of *Justicia tranquebariensis* whole plant for the turbidity method. While Cystone a

prescribed medicine for renal calculi showed highest inhibition (**76.65%**) in terms of formation of calcium oxalate precipitation **(Table.4** and**Fig.5)**.

The values are depicted in the table and the IC_{50} value of ethanolic extract of *Justicia tranquebariensis* whole plant showed significant IC_{50} value (**600.75µg/ml**) than the hexane (**842.3µg/ml**) and ethyl acetate **(796µg/ml)** extract of *Justicia tranquebariensis* whole plant. The IC_{50} value of the Cystone standard (**546.8 µg/ml**) **(Fig.6).**

Kidney oxalate stone is the result of supersaturation of urine with certain urinary salts such as calcium oxalate. Since crystallisable oxalate species are pH independent, the crystallization of oxalate in the absence of an inhibitor, led to the formation of calcium oxalate monitored by light microscope the process of calcium oxalate crystallization in control without the addition of inhibitors is shown in (**Fig.7**).The % inhibition of turbidity (aggregation) in the presence of herb extracts was lower than in the control, showing that crystals were less aggregated. The inhibited aggregation associated with the extract increased with concentration. This inhibition was greatest with ethanolic extract of whole plant of *Justicia tranquebariensis*. (**Fig.7**)

Ethanolic extract has a greater capacity to reduce all these crystallisation process as compared to hexane and ethyl acetate extract.

The ethanolic extract of *Justicia tranquebariensis* whole plant showed higher (**58.2%)** percentage dissolution of calcium oxalate than the hexane (**39.2%**) and ethyl acetate (**46.8%**) extract of *Justicia tranquebariensis* whole plant. While Cystone a prescribed medicine for renal calculi showed highest percentage dissolution (**65%**) of calcium oxalate **(Fig.8**and values depicted in the **table5)** by the Titrimetry method.

***IN-VIVO* ANTIUROLITHIATIC METHOD**

From the acute toxicity study, the dose 2000mg/kg founded as non toxic. Hence the therapeutic dose was taken as 200 mg/kg/b.w. and 400mg/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[75].

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.

The glomerular filtration rate (GFR) is an important parameter for ensuring renal function it gets decreased in urolithiasis due to the obstruction to the outflow of the urine by stone in urinary system, which leads to decreased in urine volume. Urolithiasis induced animals shows decreased in urine volume (Group II), whereas the treated groups (II, III, IV) shows increased in urine volume when compared with Group II rats (**Table 6** and **fig.9**).

In ethylene glycol induced rats urine pH was significantly reduced when compared to normal animals and treatment with plant extract was found to be significantly increased in a dose dependent manner.

In the present study, chronic administration of 0.75% v/v ethylene glycol aqueous solution for 28 days resulted in hypercalciuria in rats. Phosphorus, oxalate and calcium excretion were significantly increased ($p<0.05$), whereas magnesium decreased in urine of EG treated animals (group II) as compared to group I. However, supplementation with plant extracts at 200 mg/kg and 400mg/kg body weight and cystone 750 mg/kg body weight significantly ($P < 0.05$) lowered the elevated level of phosphorus, oxalate and calcium in urine as compared to the group II animals. Magnesium level in the standard and test groups brought back to normal level. 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 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 and Blood Urea Nitrogen (BUN). However, treatment with plant extract restored the serum levels of creatinine, uric acid and BUN. The ethanolic plant extract (Group IV and V) and Cystone (Group III) significantly ($p<0.05$) reduced the elevated serum uric acid level as compared to group II. (**Table 8)**

HISTOPATHOLOGICAL EXAMINATION OF THE RAT KIDNEYS

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 and serum creatinine that was lowered in animals receiving plant extract. 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 the presence of flavonoids constituents of the plant restore the renal antioxidant enzyme and prevent renal cell injury.

9. CONCLUSION

- From the present study we conclude that the preliminary phytochemical analysis of *Justicia tranquebariensis*. indicated the presence of Flavonoids, quinones, glycosides, coumarins and steroids.
- *In-vitro* Calcium oxalate crystallization inhibition study was evaluated. From this study conclude that the ethanol, ethyl acetate, and hexane extracts of *Justicia tranquebariensis*. inhibits the calcium oxalate crystallization in the order of 58%, 46% and 39% respectively.
- *In-vitro* turbidity method was evaluated. From this study conclude that the ethanol, ethyl acetate, and hexane extracts of *Justicia tranquebariensis*. inhibits the calcium oxalate nucleation in the order of 73%, 62% and 61% respectively.
- Among the various extracts, the ethanolic extract produce more potent anti urolithiatic activity.
- In *In-vivo* Anti urolithiatic activity of ethanolic extract of *Justicia tranquebariensis*. was evaluated and we conclude that ethanolicextracts of the plant *Justicia tranquebariensis*. produced significant antiurolithiatic activity like that of standard.

- In the present study provided basic evidence that the *justicia tranquebariensis* has the beneficial effect for the treatment of urolithiasis.

- Further studies are required to elucidate the molecular mechanism of action and its therapeutic potential in the treatment of urolithiasis.

10.REFERENCES

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