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Antiurolithic Activity of Syzygium Cumini on Ethylene Glycol Induced Kidney Stones in Male Albino Rats

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A B S T R A C T
The kidney stone is one of the most widely spreading renal disorders in the world. The present study was undertaken to evaluate the efficacy of *SCYZYGIUM CUMINI* seeds in reducing the growth of calcium oxalate stones in ethylene glycol induced model. Upon administration of Furosemide (20mg/kg), alcoholic extract of *SCYZYGIUM CUMINI* (200 and 400mg/kg) on hyperoxaluria rats shows the significant activity in decrease kidney stones and serum levels (calcium, phosphorous, Creatinine, urea) both not that as standard drug Furosemide.
Key words: *Syzygium cumini*, calcium oxalate, ethylene glycol, Furosemide, hyperoxaluria.

A R T I C L E I N F O

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CONTENTS

1. Introduction

Urinary stone disease occurs worldwide with some geographical and racial variation and is constantly rising in parallel with socio-economic development[1] .It is largely a recurrent disease with an approximate relapse rate of 50% in 5-10 years and 75% in 20 years. Urolithiasis is a common urologic disease that affects approximately 10 % population worldwide. In India, 12% of the population is expected to have urinary stones, out of which 50% may end up with loss of kidneys or renal damage. Also, nearly 15% of the population of northern India suffers from kidney stones. Fewer occurrences of urinary calculi are found in southern India. In India, the "stones belt" occupies parts of Maharashtra, Gujarat, Punjab, Haryana, Delhi and Rajasthan. In these regions, the disease is so prevalent that most of the members of a family will suffer from kidney stones sometime in their lives. Urinary tract stones composed of calcium oxalate (coax), either alone or mixed with calcium phosphate, are hitherto the most common uroliths accounting for more than 80% of stones[3]. the crystallization inhibition capacity of urine does not allow

urolithiasis to happened in most of individuals, whereas, this natural inhibition is in deficit in stone formers[4].Studies have also that tubular cell injury facilitates calcium oxalate crystal formation and deposition in renal tubules[5].animal and tissue culture studies have demonstrated that both oxalate and calcium oxalate crystals directly induce renal epithelial cell injury mediated through lipid peroxidation and involve oxygen free radical generation [6,7]. Endoscopic stone removal and extracorporeal shock wave lithotripsy have revolutionized the treatment of nephrolithiasis, but do not avoid the possibility of new stone formation [8, 9.] Various therapies including thiazide diuretics and alkali-citrate are being used in an attempt to prevent the recurrence of hypercalciuria and hyperoxaluria induced calculi, but scientific evidence for their efficacy is less convincing.

Medical plants have played as significant role in various ancient traditional system of medication. Even today, plants provide a cheap source of drugs for majority of world's population. Several pharmacological investigations on the medicinal plants used in traditional antiurolithiatic therapy have revealed their therapeutic potential in the in-vitro or in-vivo models[11, 12]. Researchers are also trying to isolate potent phytoconstituents and antiurolithiatic potency is being evaluated. The most active protein fractions are isolated from dolichos biflorus [13] and trachyspermum ammi[14] and their therapeutic use as antilithiatic protein was established.

Kidneys:

The kidneys are organs that serve several essential regulatory roles in most animals, including vertebrates and some invertebrates. They are essential in the urinary system and also serve homeostatic functions such as the regulation of electrolytes, maintenance of acid–base balance, and regulation of blood pressure (via maintaining salt and water balance). They serve the body as a natural filter of the blood, and remove wastes which are diverted to the urinary bladder. The kidneys excrete wastes such as urea and ammonium, and they are also responsible for the reabsorption of water, glucose, and amino acids.

Common clinical conditions involving the kidney include the nephritic and nephrotic syndromes, renal cysts, acute kidney injury, chronic kidney disease, urinary tract infection, nephrolithiasis, and urinary. Various cancers of the kidney exist; the most common adult renal cancer is renal cell carcinoma. Although they are not severely harmful, kidney stones can be painful and a nuisance. One common symptom of kidney stones is a sharp pain in the medial/lateral segments of the lower back.

Anatomy

Location:

In humans the kidneys are located in the abdominal cavity, more specifically in the paravertebral gutter and lie in a retroperitoneal position at a slightly oblique angle. There are two kidneys. One is on each side of the spine. The asymmetry within the abdominal cavity caused by the liver typically results in the right kidney being slightly lower than the left, and left kidney being located slightly more medial than the right[24]. The left kidney is approximately at the vertebral level T12 to L3[25], and the right slightly lower. The right kidney sits just below the diaphragm and posterior to the liver, the left below the diaphragm and posterior to the spleen. Resting on top of each kidney is an adrenal gland. The upper (cranial) parts of the kidneys are partially protected by the eleventh and twelfth ribs, and each whole kidney and adrenal gland are surrounded by two layers of fat (the perirenal and pararenal fat) and the renal fascia. Each adult kidney weighs between 125 and 170 grams in males and between 115 and 155 grams in females. The left kidney is usually slightly larger than the right kidney.

Structure:

The kidney has a bean-shaped structure; each kidney has a convex and concave surface. The concave surface, the renal hilum, is the point at which the renal artery enters the organ, and the renal vein and ureter leave. The kidney is surrounded by tough fibrous tissue, the renal capsule, which is itself surrounded by perinephric fat, renal fascia (of Gerota) and paranephric fat. The anterior (front) border of these tissues is the peritoneum, while the posterior (rear) border is the transversalis fascia. The superior border of the right kidney is adjacent to the liver; and the spleen, for the left kidney. Therefore, both move down on inhalation.

The kidney is approximately 11–14 cm in length, 6 cm wide and 4 cm thick. The substance, or parenchyma, of the kidney is divided into two major structures: superficial is the renal cortex and deep is the renal medulla. Grossly, these structures take the shape of 8 to 18 cone-shaped renal lobes, each containing renal cortex surrounding a portion of medulla called a renal pyramid (of Malpighi). Between the renal pyramids are projections of cortex called renal columns . Nephrons, the urine-producing functional structures of the kidney, span the cortex and medulla. The initial filtering portion of nephrons is the renal corpuscle, located in the cortex, which is followed by a renal tubule that passes from the cortex deep into the medullary pyramids. Part of the renal cortex, a medullary ray is a collection of renal tubules that drain into a single collecting duct.The tip, or papilla, of each pyramid empties urine into a minor calyx; minor calyces empty into major calyces, and major calyces empty into the renal pelvis, which becomes the ureter. At the hilum, the ureter and renal vein exit the kidney while the renal artery enters. Surrounding these structures is hilar fat and lymphatic tissue with lymph nodes. The hilar fat is contiguous with a fat-filled cavity called the renal sinus. The renal sinus collectively contains the renal pelvis and calyces and separates these structures from the renal medullary tissue.

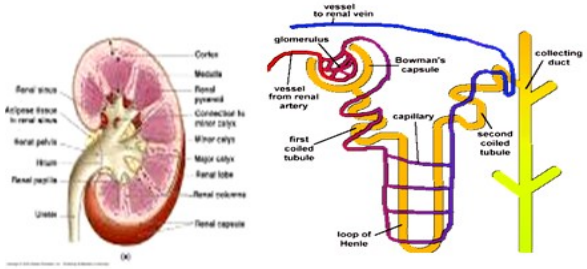


Fig 1: Structure of Kidney and Nephron

Blood Supply:

The kidneys receive blood from the renal arteries, left and right, which branch directly from the abdominal aorta. Despite their relatively small size, the kidneys receive approximately 20% of the output. Each renal artery branches into segmental arteries, dividing further into interlobar arteries which penetrate the renal capsule and extend through the renal columns between the renal pyramids. The interlobar arteries then supply blood to the arcuate arteries that run through the boundary of the cortex and the medulla. Each arcuate artery supplies several interlobular arteries that feed into the afferent arterioles that supply the glomeruli. The interstitium is the functional space in the kidney beneath the individual filters (glomeruli) which are rich in blood vessels. The interstitium absorbs fluid recovered from urine. Various conditions can lead to scarring and congestion of this area, which can cause kidney dysfunction and failure. After filtration occurs the blood moves through a small network of venules that converge into interlobular veins. As with the arteriole distribution the veins follow the same pattern, the interlobular provide blood to the arcuate veins then back to the interlobar veins which come to form the renal vein exiting the kidney for transfusion for blood.

Histology:

Renal histology studies the structure of the kidney as viewed under a microscope. Various distinct cell types occur in the kidney, including:

- Kidney glomerulus parietal cell
- Kidney glomerulus podocyte
- Kidney proximal tubule brush border cell
- Loop of Henle thin segment cell
- Thick ascending limb cell
- Kidney distal tubule cell
- Kidney collecting duct cell
- Interstitial kidney cells
- Renal arteries and their branches

Innervation:

The kidney and nervous system communicate via the renal plexus, whose fibers course along the renal arteries to reach each kidney[28]. Input from the sympathetic nervous system triggers vasoconstriction in the kidney, thereby reducing renal blood flow[28]. The kidney also receives input from the parasympathetic nervous system, by way of the renal branches of the vagus nerve (cranial nerve X).

Functions:

Maintaining homeostasis of a large number of solutes and water is the main job of the kidney. Total body contents stay normal even if dietary intake or endogenous production changes.

A. Components of the body that are regulated by the kidney

a) Electrolytes
- ✓ Sodium, the main osmole in the extracellular space
- ✓ Potassium, the major intracellular cation
- ✓ Chloride the major extracellular anion

b) Total body water (osmolality)

c) pH
- ✓ By excreting hydrogen ions
- ✓ By regulating the concentration of HCO3-, the major extracellular buffer

d) Minerals
- ✓ Calcium
- ✓ Phosphorus
- ✓ Magnesium

e) Endogenously produced waste materials
- ✓ Urea- major end product of protein catabolism
- ✓ Creatinine- produced by skeletal muscle
- ✓ Uric acid- nucleic acid breakdown product

B. The kidney also performs a number of endocrine functions.

a) The kidney is the sole source of erythropoietin
- ✓ Released in response to hypoxia; necessary to mobilize iron in bone marrow to produce haemoglobin for red blood cell production.
- ✓ Therefore, reduced number of functional nephrons leads to less erythropoietin low reticulocyte count; normocytic, normochromic anaemia.
- ✓ The anaemia caused by kidney failure can be corrected by administration of exogenous erythropoietin.

b) Kidney is also the only significant site of production of 1-hydroxylase.
- ✓ The final enzyme necessary to produce the active component of the vitamin - D system, 1, 25-(OH) 2D3.
- ✓ Loss of renal mass leads to lack of active vitamin D and thus hypocalcaemia.

c) Also the sole source of renin.

C. There are also a number of paracrine substances in the kidney that regulate homeostasis.

a) Bradykinin

b) Prostaglandins (esp. PGE2 and PGI2, natriuretic and vasodilatory)

c) Endothelial factor
- ✓ NO, this causes vasodilatation and natriuresis
- ✓ Endothelia
  - ✓ Also produced by endothelial cells, but usually only in response to injury
  - ✓ Most potent vasoconstrictor known

D. The kidney is a critical organ in the maintenance of normal blood pressure
- ✓ Regulates water and sodium, so controls blood volume.
- ✓ Controls renin-angiotensin-aldosterone axis.
- ✓ Produces some vasodilatory substances.

E. Involved in catabolism of small peptide hormones such as insulin

F. Can produce glucose via gluconeogenesis during fasting

G. Responsible for elimination of many drugs based on concentration.

H. The concept of balance
- ✓ Neutral balance means dietary intake + endogenous production excretionrate by the kidney. Total body content of the solute stays stable.
- ✓ Positive balance means intake + endogenous production > excretion.
- ✓ Total body content increases

- ✓ Negative balance means intake + endogenous production < excretion.
- ✓ Total body content decreases[29].

Kidney Diseases and Disorders Hereditary Disorders

These can transmitted to both males and females and generally produce clinical symptoms from teenage years to adulthood.

- Polycystic kidney disease.
- Alprot's syndrome.
- Primary hyperoxaluria and cystinuria.

Congenital Disease:

This usually involves some malformation of the genito-urinary tract,usually leading to some type of obstruction which subsequently produces infection or destructions of kidney tissue.The destruction can eventually progress to chronic kidney failure.

- ✓ Congenital Hydronephrosis.
- ✓ Congenital obstruction of urinary tract.
- ✓ Duplex kidneys, or double kidneys, occur in approximately 1% of the population.
- ✓ Duplicated ureter occurs in approximately one in 100 live births.
- ✓ Horseshoe kidney occurs in approximately one in 400 live births.
- ✓ Renal agenesis. Failure of one kidney to form occurs in approximately one in 750 live births. Failure of both kidneys to form is invariably fatal.
- ✓ Renal dysplasia.
- ✓ Unilateral small kidney.
- ✓ Multicystic dysplastic kidney occurs in approximately one in every 2400 live births.
- ✓ Ureteropelvic Junction Obstruction or UPJO; although most cases appear congenital, some appear to be an acquired condition.

Acquired Diseases:

These diseases are numerous, the general term being nephritis(inflammation of the kidney).

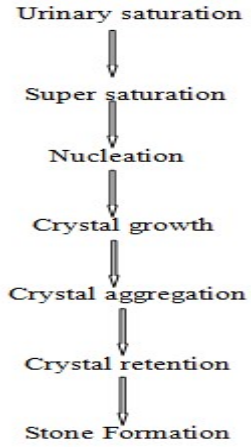
- ✓ Glomerulonephritis
- ✓ Hydronephrosis is the enlargement of one or both of the kidneys caused by obstruction of the flow of urine.
- ✓ Nephrotic syndrome, the glomerulus has been damaged so that a large amount of protein in the blood enters the urine. Other frequent features of the Nephrotic syndrome include swelling, low serum albumin, and high cholesterol.
- ✓ Diabetic nephropathy
- ✓ Interstitial nephritis
- ✓ Kidney stones (nephrolithiasis) are a relatively common and particularly painful disorder.
- ✓ Kidney tumors (Wilms tumor, Renal cell carcinoma)
- ✓ Pyelonephritis is infection of the kidneys and is frequently caused by complication of a urinary tract infection.

Urolithiasis:

Urolithiasis 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[30] refers to stones that are in the ureter. The term Cystolithiasis (or *vesicle calculi*) refers to stones which form or have passed into the urinary bladder.

Kidney stones result from crystals in the urine aggregating together when the urine becomes highly concentrated. Normally these crystals pass through the urinary tract without problems. Occasionally, if these stones become large enough (at least 3 mm (0.12 inches), they cause obstruction of the ureter. Ureteral obstruction causes 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 typically comes in waves lasting 20 to 60 minutes, beginning in the flank or lower back and often radiating to the groin or genitals.

The sequence of events in the formation of urinary stones:



Types of kidney stones:

Calcium stones: {occurring about 75 -80 % of cases} 31

When calcium combines with another mineral, insoluble crystals form which are commonly either calcium oxalate or calcium phosphate in composition. These stones can typically be seen on plain x-ray. Usually, no specific cause is found on why these stones develop, however they can occur in certain medical conditions such as hyperparathyroidism, certain types of weight reduction surgery, and in several types of kidney disorders.

Fig 2: Calcium Stones

Uric acid stones: {comprises about 5-10% of cases} [31]

These form crystals in the urine, either alone or with other stone types. They are commonly due to an excessively high protein diet, obesity, or in patients who suffer with gout. Typically, these stones form in acidic urine (pH 5-6) and are not visible on plain x-ray.

Fig 3: Uric acid stones

Struvite (Infection stones): Occurring about 10-15% of cases} [31]. They are combination of magnesium, ammonium, and phosphate. These stones can grow very rapidly. They are usually associated with urinary tract infections, which change the urinary environment to permit rapid stone growth. Consequently, the stone formed can become very large in size. If left untreated they can cause chronic infection, destroy the kidney, and may result in death.

Fig 4: Infection stones

Cysteine stones: {occurring in 1% of stone patients} 31. These are rare stones, due to an inherited defect in amino acid transport within the kidney. An excess of cysteine crystals are found in the urine of affected patients which clump together to form stones. Patients who are affected tend to be young and develop recurrent kidney stones throughout life. Long term treatment involves close surveillance, education, dietary changes, fluids, and sometimes medications to prevent the stones from recurring.

Fig 5: Cysteine stones

Causes of Kidney Stones:

- The leading cause of kidney stones is a lack of water. When there is not enough water to dilute the uric acid (component of urine), the pH level within the kidneys drops and becomes more acidic. An excessively acidic environment in the kidneys is conducive to the formation of kidney stones.
- Medical conditions such as Crohn's disease, urinary tract infections, renal tubular acidosis, hyperparathyroidism, medullary sponge kidney, have been known to lead to kidney stones.
- People who take supplemental calcium have a higher risk of developing kidney stones, by increasing urinary calcium excretion, high dietary sodium may increase the risk of stone formation. Fluoridation of drinking water may increase the risk of kidney stone formation by a similar mechanism. ,
- High dietary intake of potassium appears to reduce the risk of stone formation because potassium promotes the urinary excretion of citrate, an inhibitor of urinary crystal formation. High dietary intake of magnesium also appears to reduce the risk of stone formation somewhat, because like citrate, magnesium is also an inhibitor of urinary crystal formation.
- Diets containing large proportion of animal protein. Urinary excretion of excess sulphurous amino acids (e.g., cysteine and methionine), uric acid and other acidic metabolites from animal protein acidifies the urine, which promotes the formation of kidney stones.
- Ingestion of vitamin C supplements is associated with an increased incidence of kidney stones; excess dietary intake of vitamin C might increase the risk of calcium oxalate stone formation.
- Alcohol consumption can lead to systemic dehydration, which in turn lead toKidney stone formation.32

Signs and Symptoms of Kidney Stones:

A kidney stone usually remains symptomless until it moves into the ureter. When symptoms become apparent, they include:

- Severe pain in the groin and/or side
- Blood in urine
- Vomiting and nausea
- White blood cells or pus in the urine
- Reduced amount of excreted urine excreted
- Burning sensation during urination
- Persistent urge to urinate
- Fever and chills if there is an infection[32]

Diagnosis of Kidney Stones:

Several different tests can verify the existence of a kidney stone.

- A physical examination may reveal colicky pain (in the groin) and pain in the lower back by the kidneys - often warning signs of the condition.
- An analysis of the urine will indicate whether or not there is blood in the urine and if there is a subsequent infection.
- Blood tests can be done to identify complications that may accompany a kidney stone and check the validity of the diagnosis.
- The definitive diagnosis of a stone is made by imaging tests; A CT scan of the abdomen is the most

thorough way to test for kidney stones. A CT scan will ascertain the state of the ureter, bladder, and kidneys, whether or not a stone exists, the kidney stone's exact size and location, whether or not a blockage exists, and the state of the other adjacent organs such as the appendix, aorta, and pancreas. Pregnant women may receive an ultrasound rather than a CT scan in order to avoid unnecessary radiation.

- Once a patient is diagnosed with a kidney stone, simple x-rays will be used to track the stone's progress through the excretory system[32].

Treatment of Kidney Stones:

- One of the recognized medical therapies for prevention of stones is the thiazide and loop diuretics, such as chlorthalidone or indapamide. These drugs inhibit the formation of calcium-containing stones by reducing urinary calcium excretion.
- Allopurinol (Zyloprim) for people with hyper uricosuria and calcium stones. The drug is also used in people with gout or hyperuricemia (high serum uric acid levels). Allopurinol interferes with the production of uric acid in the liver.
- Management of pain often requires intravenous administration of NSAIDs or opioids. Orally administered medications are often effective for less severe discomfort. The mainstay for medical management of uric acid stones is alkalinisation (increasing the pH) of the urine. Acetazolamide (Diamox) is a medication that alkalinizes the urine.
- Anti-emetic medication can treat a patient suffering from nausea and vomiting. The use of medications to speed the spontaneous passage of ureteral calculi is referred to as medical expulsive therapy alpha adrenergic blockers (such as tamsulosin) and calcium channel blockers (such as nifedipine), have been found to be effective.
- Extracorporeal shock wave lithotripsy (ESWL) is a non-invasive technique for the removal of kidney stones. High-intensity pulses of ultrasonic energy cause fragmentation of a stone. Patients with large stones located in regions that do not allow for lithotripsy may receive surgical procedures such as percutaneous nephrolithotomy (removal of the stone through an incision in the back) or ureteroscopic stone removal (removal of the stone through a thin tube into the urethra).
- Ureteroscopy involves the placement of a ureteral stent (a small tube extending from the bladder, up the ureter and into the kidney) to provide immediate relief of an obstructed kidney. More definitive ureteroscopic techniques for stone extraction (rather than simply bypassing the obstruction) include basket extraction, ultrasound ureterolithotripsy. Laser lithotripsy is another technique, which involves the use of laser to fragment stones in the bladder, ureters, and kidneys[32].

Varoius ways of induction of kidney stones for experimental study: There are several methods that can be used to induce nephrolithiasis in animals especially in mice and rats. Nephrolithiasis induction using Ethylene glycol in rats by administrating 0.75% Ethylene glycol in drinking water for 28 days. Ethylene glycol (EG) is the dihydroxy alcohol derivative of ethane. The characteristic of EG are clear, colourless, sweet-tasting liquid is rapidly and completely absorbed upon ingestion with peak blood levels occurring in 1-4 hours. Ethylene glycol is filtered by glomerulus and is passively reabsorbed. Approximately 20% of ethylene glycol is excreted unchanged in the urine. When EG were metabolized by the body, it will produces four toxic metabolites, there are glyceraldehydes, glycolate, glycolic acid, and also glyoxylate. All of these metabolites may causes destruction of the tissue that primarily from calcium oxalate crystal deposition, and metabolic abnormalities, especially a high anion-gap metabolic acidosis, lactic acidosis, and hypocalcaemia. Oxalic acid combines with calcium to form calcium oxalate crystals, which deposit in the kidneys. This can result in hypocalcaemia, hematuria, and proteinuria, increased Creatinine and renal failure Induction of nephrolithiasis in mice using glyoxylate can be carry out by administrated intra-abdominal injection of glyoxylate with dose 80mg/Kg body weight until 9 days. Kidney stone can be induced by medications used to treat a variety of conditions. These medications will lead to metabolic abnormalities that facilitate formation of kidney stones. Drugs that induce metabolic calculi include loop diuretics, carbonic anhydrase inhibitors, and laxatives. Kidney stone can also be induced by medications when the drugs crystallize and become the primary component of the stones. Drugs that induce calculi via this process include magnesium trisilicate, ciprofloxacin, sulfa medications, triamterene, indinavir, and ephedrine, alone or in combination with guaifenesin.

In addition to using chemical compounds, nephrolithiasis induction in animals could also used Porang tuber powder (*Amorphophallus muelleri*) with dose 6 mg / 100 g body weight for three months. *A. muelleri* are plants with tuber that have high amount of have calcium oxalate. So, this plant can be used to induce nephrolithiasis in rat and mice.
The nephrolithiasis parameters can be determined by the accumulation of calcium oxalate in the kidneys with histological observation using specific staining for calcium oxalate. The staining that can be used to stain the accumulation calcium oxalate, there are alizarin red's staining, pizzolato's staining, and Von Kossa's staining. It's also needs to be measured the levels of Creatinine, free radicals, calcium, phosphate, and oxalate[33].

Plant Profile

Scyzygium Cumimi

Kingdom: Plantae
Unraked: Angiosperms
Unraked: Eudicots
Unraked: Rosids
Order: Myrtales
Family: Myrtaceace
Genus: Syzygium
Species: *S. Cumin*

Botanical Source:

Syzygium cumini, known as jambul, jambolan, jamblang or jamun, is an evergreen tropical tree in the flowering plant family Myrtaceae.

Distubution:

Syzygium cumini is native to the Indian Subcontinent and adjoining regions of Southeast Asia. The species ranges across India, Bangladesh, Pakistan, Nepal, Sri Lanka, Malaysia, the Philippines, and Indonesia.

Description:

A slow growing species, it can reach heights of up to 30 m and can live more than 100 years. Its dense foliage provides shade and is grown just for its ornamental value. At the base of the tree, the bark is rough and dark grey, becoming lighter grey and smoother higher up. The wood is water resistant. Because of this it is used in railway sleepers and to install motors in wells. It is sometimes used to make cheap furniture and village dwellings though it is relatively hard to work on.The leaves which are an aroma similar to turpentine, are pinkish when young, changing to a leathery, glossy dark green with a yellow midrib as they mature. The leaves are used as food for livestock, as they have good nutritional value

Syzygium cumini trees start flowering from March to April. The flowers of are fragrant and small, about 5 mm in diameter. The fruits develop by May or June and resemble large berries; the fruit of Syzygium species is described as "drupaceous".[8] The fruit is oblong, ovoid, starts green and turns pink to shining crimson black as it matures. A variant of the tree produces white coloured fruit. The fruit has a combination of sweet, mildly sour and astringent flavour and tends to colour the tongue purple.

Selection and Storage:

Seeds of Syzygium cumini with 43.6% moisture content, treated with (i) Octave [prochloraz-manganese complex] (dusting), (ii) glue (trade name Sepiret), (iii) Octave in glue, (iv) benomyl (dusting), (v) benomyl in glue, (vi) sodium hypochlorite (1%) for 10 min, and (vii) mixing with vermiculite, and without any treatment, were stored in untied polyethylene bags in an incubator at 16°C (±1°C). Untreated seeds showed significant reduction in germination after 8 weeks storage which, however, did not decline significantly further up to 20 weeks. After 20 weeks, total germination was high in all the treatments, including control. Sodium hypochlorite treatment significantly improved the speed of and total germination of stored seeds. Total germination of seeds with and without treatment with benomyl in glue and stored for 20 weeks at 5°C was also high and at par with the treated and untreated seeds stored at 16°C. Speed of germination of untreated seeds stored at the two temperatures was at par after 8 weeks, but after 12 weeks storage, seeds stored at 16°C germinated significantly faster than those stored at 5°C

Chemical Constituents and Uses:

The seed is also used in various alternative healing systems like Ayurveda (to control diabetes, for example), Unani and Chinese medicine for digestive ailments.The pulp of the fruit, extracts from the bark and seeds is of great benefit when it comes to lowering of blood glucose level. Taking dried extract of the seeds orally greatly reduces the blood sugar and glucosuria.The leaves and bark are used for controlling blood pressure and gingivitis. Wine and vinegar are also made from the fruit. It has a high source in vitamin A and vitamin C.Syzygium cumini has been spread overseas from India by Indian emigrants and at present is common in former tropical British colonies.

Standard Drug Profile:

Furosemide:

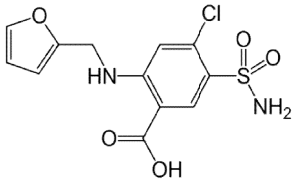


Fig 6: Structure of Furosemide

Furosemide is used here as a standard drug. It belongs to the category of Loop diuretic. It also possesses potential anti-urolithic activity due to its diuretic character.

Mechanism of Action:

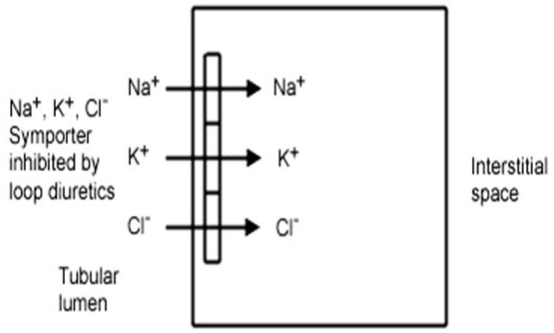


Fig 7: Mechanism of action of Furosemide

Mechanism of Action of Furosemide:

Furosemide, like other loop diuretics, acts by inhibiting NKCC2, the luminal Na-K-2Cl symporter in the thick ascending limb of the Loop of Henle. The action on the distal tubules is independent of any inhibitory effect on carbonic anhydrase or aldosterone; it also abolishes the corticomedullary osmotic gradient and blocks negative, as well as positive, clearance. Because of the large NaCl absorptive capacity of the loop of Henle, diuresis is not limited by development of acidosis, as it is with the carbonic anhydrase inhibitors.

By inhibiting the transporter, the loop diuretics reduce the reabsorption of NaCl and also diminish the lumen-positive potential that derives from K^+ recycling. This electrical potential normally drives divalent cation reabsorption in the loop, and by reducing this potential, loop diuretics cause an increase in Mg^{2+} and Ca^{2+} excretion. Prolonged use can

cause significant hypomagnesaemia in some patients. Since Ca^{2+} is actively reabsorbed in the distal convoluted tubule, loop diuretics do not generally cause hypocalcaemia.

Additionally, Furosemide is a non-competitive subtype-specific blocker of GABA-A receptors. Furosemide has been reported to reversibly antagonize GABA-evoked currents of $\alpha_6\beta_2\gamma_2$ receptors at μM concentrations, but not $\alpha_1\beta_2\gamma_2$ receptors. During development, the $\alpha_6\beta_2\gamma_2$ receptor increases in expression in cerebella granule neurons, corresponding to increased sensitivity to Furosemide

Pharmacokinetics:

The pharmacokinetics of Furosemide has been studied in a limited fashion in domestic animals. In dogs, the oral bioavailability is approximately 77% and the elimination half-life approximately 1 – 1.5 hours. In humans, Furosemide is 60 – 75% absorbed following oral administration. The diuretic effect takes place within 5 minutes after I.V administration and within one hour after oral dosing. Peak effects occur approximately 30 minutes after I.V dosing, and 1 -2 hours after oral dosing. The drug is approximately 95% bound to plasma proteins in both azotemic and normal patients. The serum half-life is about 2 hours, but prolonged in patient with renal failure, uremia, Congestive heart failure, and in neonates.[40]

Pharmacodynamics

Effects on Urinary Excretion:

Owing to blockade of the Na^+-K^+-$2Cl^-$ symporter, Furosemide increase in the urinary excretion of Na^+ and Cl^- profoundly (i.e., up to 25% of the filtered load of Na^+).Abolition of the transepithelial potential difference also results in marked increases in the excretion of Ca^{2+} and Mg^{2+}.Furosemide has weak carbonic anhydrase-inhibiting activity. All inhibitors of Na^+-K^+-$2Cl^-$ symport increase the urinary excretion of K^+ and titratable acid. This effect is due in part to increased delivery of Na^+ to the distal tubule.

Other mechanisms contributing to enhanced K^+ and H^+ excretion include flow-dependent enhancement of ion secretion by the collecting duct, non-osmotic vasopressin release, and activation of the renin-angiotensin-aldosterone axis (Wilcox, 1999).Acutely, loop diuretics increase the excretion of uric acid, whereas chronic administration of these drugs results in reduced excretion of uric acid. The chronic effects of loop diuretics on uric acid excretion may be due to enhanced transport in the proximal tubule secondary to volume depletion, leading to increased uric acid reabsorption, or to competition between the diuretic and uric acid for the organic acid secretory mechanism in the proximal tubule leading to reduced uric acid secretion by blocking active Nacl reabsorption in the thick ascending limb, inhibitors of Na^+-K^+-$2Cl^-$ symport interfere with a critical step in the mechanism that produces a hypertonic medullary interstitium.

Therefore, loop diuretics block the kidney's ability to concentrate urine during hydropenia. Also, since the thick ascending limb is part of the diluting segment, inhibitors of Na^+-K^+-$2Cl^-$symport markedly impair the kidney's ability to excrete dilute urine during water diuresis.[41]

Effects on Renal Hemodynamics:

Nonsteroidal anti-inflammatory drugs (NSAIDs) attenuate the diuretic response to loop diuretics in part by preventing prostaglandin-mediated increases in RBF. Loop diuretics block TGF by inhibiting salt transport into the macula densa so that the macula densa no longer can detect NaCl concentrations in the tubular fluid. Therefore, unlike carbonic anhydrase inhibitors, loop diuretics do not decrease GFR by activating TGF. Loop diuretics are powerful stimulators of renin release. This effect is due to interference with Nacl transport by the macula densa and if volume depletion occurs, to reflex activation of the sympathetic nervous system and to stimulation of the intrarenal baroreceptor mechanism. Prostaglandins, particularly prostacyclin, may play an important role in mediating the renin- release response to loop diuretics.

Other Actions:

Furosemide may cause direct vascular effects (Dorman's et al., 1996). It acutely increases systemic venous capacitance and thereby decrease left ventricular filling pressure. This effect may be mediated by prostaglandins and requires intact kidneys, benefits patients with pulmonary edema even before dieresis ensures. Furosemide and ethacrynic acid can inhibit Na^+, K^+-ATPase, glycolysis, mitochondrial respiration ,the microsomal Ca^{2+} pump, adenylyl cyclase, phosphodiesterase and prostaglandin dehydrogenase; however these effects do not have therapeutic implications. In vitro, high doses of inhibitors of Na^+-K^+-$2Cl^-$ symport can inhibit electrolyte transport in many tissues. Only in the inner ear, where alterations in the electrolyte composition of endolymph may contribute to drug-induced Ototoxicity, is this effect important clinically.[41, 43]

Uses:

- ✓ Furosemide is used in the treatment of pulmonary edema.
- ✓ Furosemide is used widely for the treatment of chronic congestive heart failure when diminution of extracellular fluid volume is desirable to minimize venous and pulmonary congestion.
- ✓ Furosemide is used widely for the treatment of hypertension.
- ✓ Furosemide is also employed in the treatment of edema and ascites of liver cirrhosis however, care must be taken not to induce encephalopathy or hepatorenal syndrome.
- ✓ In patients with a drug overdose, Furosemide can be used to induce a forced diuresis to facilitate more rapid renal elimination of the offending drug. Loop diuretics, combined with isotonic saline administration to prevent volume depletion, are used to treat hypercalcemia.
- ✓ Furosemide combined with hypertonic saline is useful for the treatment of life-threatening hyponatremia.
- ✓ Furosemide is also used to treat edema associated with chronic renal insufficiency.
- ✓ Furosemide has the potential or capacity to remove calcium stones via urine due to its forceddiuresis.[45]

Adverse Effects:

- ✓ Adverse effects unrelated to the diuretic efficacy are rare, and most adverse effects are due to abnormalities of fluid and electrolyte balance.

- ✓ Overzealous use of loop diuretics can cause serious depletion of total-body $Na^{+.}$ It also causes extracellular fluid volume depletion associated with hypotension, reduced GFR, circulatory collapse, thromboembolic episodes.[43]
- ✓ Furosemide also causes hepatic encephalopathy in patients suffering with liver diseases.
- ✓ Increased delivery of Na^+ to the distal tubule, particularly when combined with activation of the renin-angiotensin system, leads to increased urinary excretion of K^+ and H^+ causing a hypochloremic alkalosis. If dietary K^+ intake is not sufficient, hypokalemia may develop, and this may induce cardiac arrhythmias, particularly in patients taking cardiac glycosides.[44]
- ✓ Increased Mg^{2+} and Ca^{2+} excretion may result in hypomagnesaemia (a risk factor for cardiac arrhythmias) and hypocalcaemia (rarely leading to tetany).
- ✓ Furosemide in postmenopausal osteopenic women increases Ca^{2+} excretion and has deleterious effects on bone metabolism (Rejnmark et al., 2003).
- ✓ Other adverse effects include skin rashes, photosensitivity, paresthesias, bone marrow depression, and gastrointestinal disturbances.

Toxicity:

Furosemide causes Ototoxicity manifests as tinnitus, hearing impairment, deafness, vertigo, and a sense of fullness in the ears. Hearing impairment and deafness are usually, but not always, reversible Ototoxicity occurs most frequently with rapid intravenous administration and least frequently with oral administration. Furosemide also can cause hyperuricemia (occasionally leading to gout) and hyperglycemia (infrequently precipitating diabetes mellitus) and can increase plasma levels of low-density lipoprotein (LDL) cholesterol and triglycerides while decreasing plasma levels of high-density lipoprotein (HDL) cholesterol. The acute toxicity of Furosemide has been determined in mice, rats and dogs. In all three, the oral LD_{50} exceeded 1000 mg/kg body weight, while the intravenous LD_{50} ranged from 300 to 680 mg/kg. The acute intra gastric toxicity in neonatal rats is 7 to 10 times that of adult rats.[46]

Drug Interactions:

- Amino glycosides cause synergism of ototoxicity of both the drugs.[42]
- Anticoagulants cause increase in anticoagulant activity.
- Digitalis glycosides increase digitalis-induced arrhythmias.
- Sulfonylurea's cause hyperglycemia.[47]
- Cisplatin increases risk of diuretic –induced ototoxicity.
- NSAIDs blunted diuretic response and salicylate toxicity when given with high doses of salicylates.
- Amphotericin B increases potential for nephrotoxicity and toxicity and intensification of electrolyte imbalance.

Contraindications:

Furosemide is contraindicated in patients with anuria and in patients with a history of hypersensitivity to Furosemide.

Dosage:

Hypertension- 40 mg twice a daily[48]

Edema- 20 mg – 600 mg daily taken in the form of single dose or divided doses.

Pediatrics- 2 mg per kilogram – 6 mg per kilogram.

Diuresis – 20 mg per kilogram.

Antiurolithiasis – 20 mg per kilogram.

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Scope of Study and Plan of Work

Scope of Study:

In our daily life we can see many patients suffering from urolithiasis, but the drugs available in the market are very limited. So we have taken an attempt to search for a drug from plant. Some plants with leveled medicinal values are still little known which need a very systemic scientific study to explore its exact therapeutic value. There is no proper documentation about the Antiurolithic activity of Syzygium cumini. As for the above reason the present study was carried out on male albino rats and dose fixation was done as per the protocol.

Plan of Work:

- Collection of Syzygium cumini seeds
- Extraction of various extracts by soxhlet extraction processes

- Acute toxicity study of extract of Syzygium cumini to determine the LD50 and selection of dose
- Assessment of urolithiasis activity in rats as described in protocol.
- Evaluation of Antiurolithiasis activity of Syzygium cumini .The results obtained from control and experimental animals of present investigation are compared and discussed.

2. Material and Methods

Plant Collection:

Large number of Syzygium cumini seeds was collected from the local botanical garden

Preparation of Syzygium Cumini Extract:

Alcoholic extraction was continuously done every day throughout the course of the project in order to yield a fresh extract. The powdered material was taken in beaker with water of 500ml. It was continuously heated for 18 hours. During the heating period, occasional stirring was done. After 18hrs of extraction the solution was filtered through a fine muslin cloth. The filtrate obtained is heated up to evaporation in order to obtain our desired product. The final dried samples were stored in labeled sterile bottle and kept at 20^0c.[49]

Standard Drug:

Furosemide is used as a standard drug. It leads to water loss via increased urine production and leads to the removal of kidney stones. Hence it acts as a "diuretic".

Requirements:

Cage, Feed bread, Rat Cage

Chemicals Required:

Ethylene glycol, powdered Syzygium cumini, Formaldehyde, eosin dye, Haematozylin dye, Sodium CMC, Immersion oil.

Selection of animals:

Healthy male albino rats of Wister strain weighing about 150-200 gms of equivalent age groups were obtained from central animal house of were selected for urolithiasis and toxicological studies. Chances for stone formation are more in males when compared to the females because of the absence of estrogens in males.

Maintainence of animals:

The animal house was well ventilated and animals were kept at the optimum temperatures. Rats were acclimatized for one month in polypropylene cages under hygienic conditions and provided with standard animal feed and water *ad libitum*. All procedures were done in accordance with ethical guidelines for care and use of laboratory animals and were approved by the local care of experimental animal committees.

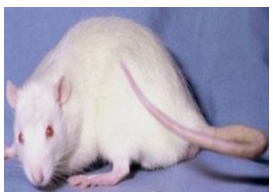

Fig 8: Albino Rat

Acute Toxicity Studies:

The aqueous extract of Syzygium cuminiwas suspended with Na-CMC administered orally in very high doses up to 2000mg/kg body weight of rats, which did not produce any toxic effects. No rats were died within 24hrs, nor have any side effects been observed.

Protocol

Induction of stone:

Ethylene glycol induced hyperoxaluria model was used to assess the Antiurolithic activity in albino male rats.0.75% v/v ethylene glycol is mixed in drinking water of rats and administered orally, At 0.75% concentration, ethylene glycol induces calcium stones in the kidney without causing any adverse effects in the rats; hence the above dose is selected. Time taken for formation of stone is 8-12 days.

Experimental Procedure:

Animals were divided into 5 groups containing 6 animals in each.

Group-1: They were fed with animal feed and drinking water *ad libitum* for 20 days. They served as a normal.

Group-2: They were fed with animal feed and drinking water *adlibitum* mixed with ethylene glycol 0.75% for 20days. They served as controlled group.

Group-3: They were fed with animal feed and drinking water *ad libitum* mixed with ethylene glycol 0.75% .Before giving the feed, they were given a dose of 20 mg/kg body weight of standard drug (Furosemide) dissolved in Na-CMC. They served as standard group.

Group-4: They were fed with animal feed and drinking water *ad libitum* mixed with ethylene glycol 0.75% .Before giving the feed, they were given a dose of 200mg/kg body weight of test drug (extract of Syzygium cumini) dissolved in Na-CMC. They served as low dose test group.

Group-5: They were fed with animal feed and drinking water *ad libitum* mixed with ethylene glycol 0.75% .Before giving the feed, they were given a dose of 400mg/kg body weight of test drug (extract of Syzygium cumini) dissolved in Na-CMC. They served as high dose test group.

Duration:

The duration of experiment was 20 days.

Treatment:

The type of treatment adopted was pre-treatment, where the dose was administered before giving the feed to rats.

3. Results and Discussion

Parameters to be evaluated

Serum Analysis: On the 19th day, the blood from each group were collected and analyzed for calcium, phosphorous, urea and Creatinine levels in the blood. The rats were anaesthetized with chloroform and blood was collected from the retro-orbital plexus region 50, 51 and centrifuged at 10000 *g for 10 min. the serum was collected and analyzed.

Serum Calcium Analysis

For the determination of calcium in serum or plasma OCPC method is employed.[52, 53]

Summary:
Calcium, in the body is found mainly in the bones (approximately 99%).In serum calcium exists equally in a free ionized form and in a bound form (with albumin).Hence a decrease in Albumin causes lower calcium levels and vice-versa. The levels of calcium in serum depend on the parathyroid hormone. Increased calcium levels are found in bone tumors, hyperparathyroidism. Decreased levels are found in hypo parathyroidism, renal failure, rickets, vitamin D deficiency and pancreatitis.

Principle:
Calcium in alkaline medium combines with o-cresolphthale to form a purple coloured complex. The intensity of the color formed is directly proportional to the amount of calcium present in the sample. The absorbance was measure at 570nm.

Normal Reference Values: Serum/plasma : 8.7 - 11mg/dl.

S.No	Contents	2×35 ml	2×75ml
1	1.1:Buffer reagent	35 ml	75 ml
2	1.2:Colour reagent	35 ml	75 ml
3	S:Calcium standard(10 mg/dl)	5 ml	5 ml

Storage/Stability: Contents are stable at 2-8^{o}C till the expiry mentioned on the labels.

Reagent Preparation:
Reagents are ready to use. Protect from bright light.

Working Reagent:
For convenience a single working reagent may be prepared by mixing equal parts of the buffer reagent and colour reagent. This combined reagent is stable for 7 days at 2-8 C.

Sample Material:
Serum/Herparinised plasma. Calcium is reported to be stable in serum for 7 days at 2-8^{o}C.

Procedure:
Wavelength/filter : 570 nm (Hg 578 nm)
Temperature : R.T.
Light path : 1 cm
Pipette into clean dry test tubes labeled as Blank (B), Standard (S), and Test (T)

S.No	Contents	B(ml)	S(ml)	T(ml)
1	Buffer Reagent(L1)	0.5	0.5	0.5
2	Colour Reagent(L2)	0.5	0.5	0.5
3	Distilled water	0.2	-	-
4	Calcium Standard(S)	-	0.02	-
5	Sample	-	-	0.02

Mix well and incubate at R.T. (25^{o}C) for 5 min. Measure the absorbance of the Standard (Abs.T) and Test Sample (Abs.T) against the Blank, within 60 min.

Calculations:
Calcium in mg/dl= (absorbance of. Test/absorbance of .standard) × 10

Linearity:
This procedure is linear up to 18mg/dl. If values exceed this limit, dilute the sample with distilled water and repeat the assay. Calculate the value using the proper dilution factor.

Note: As calcium is a widely distributed ion, care should be taken to avoid any contamination. All glass wares being used for the test they should be rinsed with 1% or 0.1 N HCL and then with good quality deionised water before use.It is suggested that after rinsing of the tubes with HCL, the reagent be pipette in their respective tubes and the tubes be rinsed with the reagent. The reagent then should be pooled together in the 'blank' tube and re-pipette out into the 'standard' and 'test' tubes. This will ensure that any remaining contamination will be carried over equally in all the tubes. For flow cell cuvettes it is suggested that some reagent be aspirated before the blank to take away any contamination in the flow through tubing or cuvettes which may cause a higher than the actual blank of the reagent. Chelating agents as EDTA, present even in traces, prevent the formation of the colour complex; hence necessary care should be taken during the assay.

System Parameters:

Reaction : End Point	Interval : -
Wavelength : 570 nm	Sample volume : 0.02 ml
Zero setting : Reagent Blank	Reagent volume : 1.0 ml
Incubation Temperature : R.T.	Standard : 10 mg/dl
Incubation Time : 5 min	Factor : -
Delay time : -	Reaction slope : Increasing
Read time : -	Linearity : 18 mg/dl
No. of read : -	Units : mg/dl

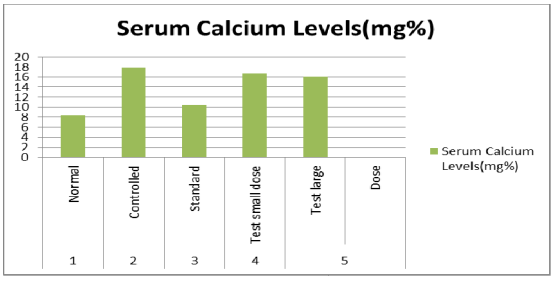

Fig 9: Graph of Serum Calcium level

Discussion:
From the (graph-1 and table 1) shows that

- In the normal group (N), the serum calcium levels are maintained at normal levels.

- In the controlled group (C), the serum calcium levels are abnormally high when compared to the other groups.
- In the standard group (S), the serum calcium levels are in similar lines with the normal groups. But it comparatively low when compared to test small dose (TSD) and test large dose (TLD).it is very low when compared to controlled group(C).
- In the test small dose group (TSD),the serum calcium levels are relatively higher when compared to that of other groups. but it is very lower when compared to the controlled group (C).
- In the test large dose group (TLD),the serum calcium levels are in similar lines with that of normal group (n)and standard group (S).it is relatively lower when compared to that of test small dose(TSD)and it is much lower than that of controlled group(C) .

Serum phosphorous analysis

For the determination of inorganic phosphorous in serum, plasma and urine Molybdate u.v method is employed[54, 55]

Summary:

Phosphorus is mainly combined with calcium and is found in the bones. Approximately 15% exists as inorganic phosphorous or phosphate esters. It is involved in the carbohydrate metabolism and is a component of many other substances. Increased levels are found in hypo-parathyroidism, renal failure, bone metastasis and liver diseases. Decreased levels are found in hyper-parathyroidism, rickets and vitamin D deficiency.

Principle:

Phosphate ions in acidic medium react with ammonium Molybdate to form a phosphomolybdate complex. This complex has an absorbance in u.v range at 340nm.intensit of complex formed is directly proportional to the amount of inorganic phosphorous present in the sample.

Phosphorous + Ammonium Molybdate - Phosphomolybdate complex

S.No	Contents	75 ml	2x75 ml
1	L1: Acidic Reagent	60 ml	2×60 ml
2	L2: Molybdate Reagent	15 ml	2× 15 ml
3	S: Phosphorous Standard(5mg/dl)	5 ml	5 ml

Storage/Stability:

Reagents are stable at R.T.(25-30^{o}c) till the expiry mentioned on the labels.

Reagent Preparation:

Reagents are ready to use.

Working Reagent:

Pour the contents of 1bottle of L2 (Molybdate Reagent into 1 bottle of L1(Acid Reagent).This working reagent is stable for at least 6 months when stored at 6 months when stored at 2-8^{o} C. Upon storage the 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 but mixing together 4 parts of L1(Acid Reagent) and 1 part of L2 (Molybdate Reagent).Alternatively 0.8 ml of L1 and 0.2ml of L2 may also be used instead of 1 ml of the working reagent directly during the assay.

Sample Material: Serum, Herparinised/ EDTA Plasma or urine. Acidify the urine with a few drops of conc. Hydrochloric acid and dilute 1+19 before the assay. (Results*20).Inorganic phosphorous is reported to be stable in serum for 7days at 2-7^{o}C.

Procedure:

Wavelength/filter : 340 (Hg 365)
Temperature : R.T.
Light path : 1 cm

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

S.No	Addition sequence	B(ml)	S(ml)	T(ml)
1	Working reagent	1.0	1.0	1.0
2	Distilled water	0.01	-	-
3	Phosphorous Standard (S)	-	-	-
4	Sample	-	0.01	0.01

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

Calculations:

Phosphorous in mg/dl= (absorbance of. Test/absorbance of standard) × 5

Linearity:

This procedure is linear up-to 20mg/dl. Measure the absorbance of the standard (Abs.S), and Test Sample (Abs) against the Blank, within 60 min.

Note:

Hemolysis interferes with the test.

Use clean glassware washed with N/10 HCL as detergents may contain phosphate ions.

System Parameters:

Reaction : UV end Point	Interval : -
Wavelength : 340 nm	Sample volume : -
Zero setting : Reagent Blank	Reagent volume : 0.01 ml
Incubation Temperature: R.T.	Standard : 40 mg/dl
Incubation Time : 5 min	Factor : 5mg/dl
Delay time : -	Reaction slope : Increasing
Read time : -	Linearity : 20 mg/dl
No. of read : -	Units : mg/dl

Discussion:

From the (graph-2 and table -1) shows that

- In normal group (N), the serum phosphate levels are maintained at normal levels.
- In the controlled group (C), the serum phosphate levels are abnormally high when compared to the other groups.
- In the standard group (S), the serum phosphate levels are in similar lines with the normal groups, but it is comparatively low when compared to test small dose (TSD)and test large dose (TLD).it is very low when compared to controlled group (C).
- In the test small dose group (TSD), the serum phosphate levels are relatively higher when compared to that of other groups. But it is very lower when compared to the controlled group (C)
- In test large dose group (TLD),the serum phosphate levels are in similar lines with that of normal group (N) and standard group (S).it is relatively lower when compared to that of test small dose (TSD)and it is much lower than that of controlled group (C).

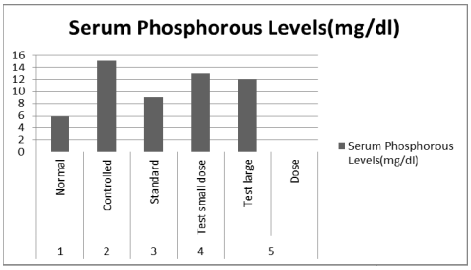

Fig 10: Serum Phosphorous level

Serum Creatinine Analysis

For the determination of Creatinine in serum and urine alkaline picrate method is used[56 , 57].

Summary:

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 of 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 and forms an orange colored complex with alkaline picrate. Intensity of color formed is directly proportional to the amount of Creatinine present in the sample and absorbance at 520 nm.

S.No	Contents	15 Tests	35 Tests
1	L1:Picric acid reagent	60 ml	140 ml
2	L2:Buffer reagent	5 ml	12 ml
3	S:Creatinine standard(2mg/dl)	5 ml	5 ml

Storage/Stability:

Reagents are stable at R.T. till the expiry mentioned on the labels.

Reagent Preparation:

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

Sample Material:

Serum or urine. Creatinine is stable in serum for 1 day at 2-8^{o}C.Urine of 24 hours collection is preferred. Dilute the specimen 1:50 with distilled/deionised water before the assay.

Procedure:

Wavelength/filter : 520nm (Hg 546nm)/Green
Temperature : 25^{o}C
Light path : 1 cm

Deprotinization of specimen:

Pipette out the following contents into the test-tube.

Picric acid reagent(L1)	2.0 ml
Sample (serum)	0.2 ml

Mix well and centrifuge at 2500-3000 rpm for 10 min to obtain a clear supernatant.

Colour Development:

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

S.No	Addition Sequence	B(ml)	S(ml)	T(ml)
1	Supernatant	-	-	1
2	Picric acid reagent(L1)	1	1	-
3	Distilled water	0.1	-	-
4	Creatinine Standard (S)	-	0.1	-
5	Buffer reagent(L2)	0.1	0.1	0.1

Mix well and keep the test tubes at R.T. for 20 min. Measure the absorbance of the Standard and Test Sample against the blank.

Calculations:

Creatinine in mg% = (absorbance of test/absorbance of standard) ×2

Urine Creatinine in gm/lit = (absorbance of test/absorbance of standard) ×1

Urine Creatinine gm/24hrs= (urine Creatinine in gm/l) × volume of urine in 24 hrs

Linearity:

The procedure is linear up to 8mg% of Creatinine. If the values exceed this limit, dilute the sample with distilled water and repeat the assay. Calculate the value using the proper dilution factor.

Note:

Maintain the reaction time of 20 min as closely as possible since a longer incubation cause an increase in the values due to the reaction of pseudochromogens. The determination is not specific and may be affected by the

presence of large quantities of reducing substances in the sample. The reaction is temperature sensitive and all the tubes should be maintained at a uniform temperature

System Parameters

Wavelength : 520 nm	Sample volume : 0.20 ml
Zero setting : Reagent Blank	Reagent volume : 1.10 ml
Incubation Time : 20 min	Factor : -
Delay time : -	Reaction slope : Increasing
Read time : -	Linearity : 8 mg/dl
No. of read : -	Units : mg/dl

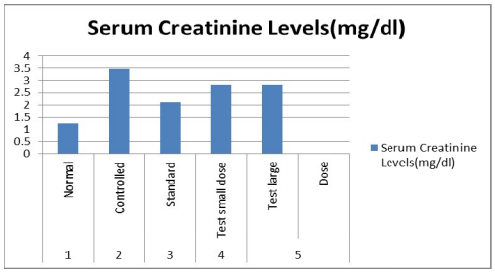

Fig 11: Serum Creatinine level

Discussion:

From the (graph-3 and table -1) shows that

- In normal group (N), the serum Creatinine levels are maintained at normal levels.
- In the controlled group (C), the serum Creatinine levels are abnormally high when compared to the other groups.
- In the standard group (S), the serum Creatinine levels are in similar line with the normal groups. But it is comparatively low when compared to test small dose (TSD) and test large dose (TLD).it is very low when compared to controlled group (C).
- In the test small dose group (TSD), the serum Creatinine levels are relatively higher when compared to that of other group. But it is very lower when compared to the controlled group (C).
- In the test large dose group (TLD), the serum Creatinine levels are in similar lines with that of normal group (N) and standard group (S).it is relatively lower when compared to that of test small dose (TSD) and it is much lower than that of controlled group (C).

Serum Urea Analysis

For the determination of urea in serum, plasma and urine moderate Berthelot method is employed[58, 59].

Summary:

Urease hydrolyses to ammonia and carbon dioxide. The ammonia formed further reacts with a phenolic chromogen and hypochlorite to form a green colored complex. Intensity of the colour formed is directly proportional to the amount of urea present in the sample.

Principle: Urease hydrolyses urea to ammonia and carbon dioxide. The ammonia formed further react with phenolic chromogen and hypochlorite to form a green colored complex. Intensity of the color formed is directly proportional to the amount of urea present in the sample. The absorbance is measured at 570 nm.

Normal Reference Values:

Serum/Plasma : 14-40 mg/dl

Urine : up to 20 g/L

S. No	Contents	75 assays	3×75 ml
1	L1:Buffer reagent	75 ml	3×75ml
2	L2:Enzyme reagent	7.5 ml	3×7.5ml
3	L3:Chromogen	15 ml	3×15ml
4	S:Standard urea (40mg/dl)	5 ml	3×5 ml

Storage/Stability:

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

Reagent Preparation:

Reagents are ready to use for the given procedure.

Working Enzyme Reagent:

For the flexibility and convenience in performing large assay series, a working enzyme reagent may be made by pouring 1 bottle of L2 (Enzyme reagent) into 1 bottle of L1 (Buffer reagent).For smaller series combine 10 parts of L1 (Buffer reagent) and 1part of L2(Enzyme reagent).Use 1 ml of the working reagent per assay instead of 1 ml of L1 and 0.1 ml of L2 as given in the procedure.

Working Chromogen Reagent:

For large volume cuvettes, dilute 1 part of L3 (Chromogen reagent) with 4parts of fresh ammonia free distilled/deionised water. Use 1ml of working chromogen instead of 0.2 ml in the assay. The working chromogen reagent is stable for at least 8 weeks when stored at 2-8^{o} C in a tightly stoppered plastic bottle.

Sample Material:

Serum, Plasma and urine. Dilute urine1+49 with distilled water before the assay. (Results*50).Urea is reported to be stable in serum for 5days when stored at 2-8^{o}C in a tightly stoppered plastic bottle.

Procedure:

Wavelength/filter : 570 (Hg 578)/Yellow.

Temperature : 37^{o} C.

Light path : 1 cm.

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

S. No	Addition sequence	B(ml)	S(ml)	T(ml)
1	Working reagent	1.0	1.0	1.0
2	Distilled water	0.01	-	-
3	Phosphorous Standard (S)	-	0.1	-
4	Sample	-	-	0.1

Mix well and incubate for 5 minutes at 37^{o}C or 10 minutes at R.T. (25^{o}C).

Chromogen Reagent (L3)	0.2	0.2	0.2

Mix well for 5 min or 10 min and incubate at room temperature. Measure the Absorbance of Standard(S), Test (T) against the Blank (B) within 60min.

Calculations:

Urea in mg/dl= (absorbance of test/absorbance of standard) × 40

Urea Nitrogen in mg/dl=Urea in mg/dl × 0.467.

Linearity: This procedure is linear up to 250 mg/dl. Using the working chromogen reagent (1ml) the linearity is increased to 400 mg/dl. If values exceed this limit, dilute the serum with normal saline (NaCl 0.9%) and repeat the assay. Calculate the value using the proper dilution factor.

Note:

Any contamination by ammonia salts lead to erroneous results, hence plasma should not be collected with Fluoride or Heparin Ammonium salts. The working enzyme reagent is not stable at elevated temperatures and should be stored back at 2- 8^{o}C immediately after use. The chromogen reagent contains chlorine. The bottle should be opened only when required and closed tightly after use to prevent the loss of active chlorine.

System Parameters

Reaction : End Point	Interval : -
Wavelength : 570 nm	Sample volume : -
Zero setting : Reagent Blank	Reagent volume : 0.01 ml
Incubation Temperature : 37^{o}C R.T.	Standard : 40 mg/dl
Incubation Time : 5 min+5min	Factor : -
Delay time : -	Reaction slope : Increasing
Read time : -	Linearity : 250 mg/dl
No. of read : -	Units : mg/dl

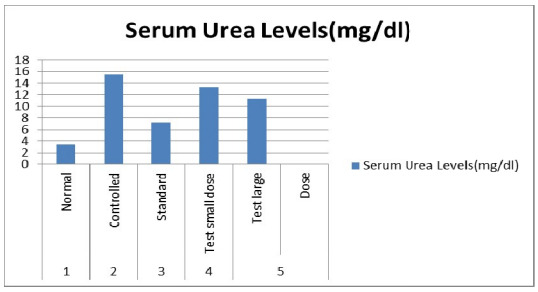

Fig 12: Graph of Serum Urea level

Discussion:

From the (graph-4 and table-1) shows that

- In the normal group (N), the serum urea levels are maintained at normal levels.
- In the controlled group (C), the serum urea levels are abnormally high when compared to other groups.
- In the standard group (S),the serum urea levels are in similar lines with the normal groups but it is comparatively low when compared to test small dose (TSD) and test large dose(TLD).it is very low when compared to controlled group (C).
- In the test small dose group (TSD), the serum urea levels are relatively higher when compared to that of other group. But it is very lower when compared to the controlled group (C).
- In the test large dose group (TLD),the serum urea levels are in similar lines with that of normal group (N) and standard group (S).it is relatively lower when compared to that of test small dose (TSD)and it is much lower than that of controlled group(C).

Kidney weights:

On the 20th day of the experiment period, all the rats were sacrificed by cervical dislocation. They were dissected by opening the abdomen and both the kidneys of each rat were removed and weighed. Increase of weight shows the formation of stones.

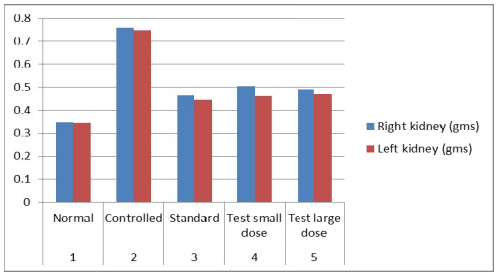

Fig 13: Graph of average weight of Kidneys

Discussion:

From the (graph-5 and table-2) shows

- The right kidney weight of the normal group (N)is the lowest when compared to that of the other groups. But the right kidney weight of the controlled group (C) is abnormally high than that of the other groups because of formation of kidney stones in them. The kidney weight of standard group (S) is at an intermediate range between normal and test groups. Both test small dose (TSD)and test large dose (TLD) groups possess similar kidney weights among each other, but their weights are more when compared to n and s groups, less when compared to that of control (C) group.
- Similar kind of inference as mentioned above can be drawn for left kidney weights in all groups of rats.

Table 1 The average of blood serum parameters along with their standard deviations for each group

S.No	Animal Treatment	Serum Calcium Levels (mg %)	Serum Phosphorous Levels(mg/dl)	Serum Creatinine Levels(mg/dl)	Serum Urea Levels(mg/dl)
1	Normal	8.298	5.938	1.237	3.424

2	Controlled	17.84	15.125	3.477	15.520
3	Standard	10.421	9.119	2.1045	7.180
4	Test small dose	16.72	12.973	2.8292	13.377
5	Test large Dose	16.007	12.045	2.8292	11.32

Table 2 The average of the kidney weights along with their standard deviation values for each group

S. No	Animal treatment	Right kidney (gms)	Left kidney (gms)
1	Normal	0.348±0.024	0.345±0.020
2	Controlled	0.758±0.125	0.745±0.098
3	Standard	0.465±0.018	0.445±0.044
4	Test small dose	0.505±0.068	0.463±0.064
5	Test large dose	0.491±0.027	0.470±0.031

Values are expressed as mean ± sem (n=6)

4. Conclusion

From the above results and study we can conclude that, Syzygium cumini is having a significant Antiurolithic activity, since it has reduced the serum levels of the above chemical constituents in the blood which were increased due to the development of stone in the kidney.

Ex: - The serum calcium levels of the normal group were 8.298 mg%. It remained under normal range, since kidney stones were not induced via diet. But in case of controlled group the serum calcium levels were 17.84 mg% which means they are at abnormal levels due to induction of kidney stones by ethylene glycol mixed in the diet. In standard group, the serum calcium levels were 10.421 mg%, which remained in similar lines with that of the normal group due to the potential Antiurolithic activity of the standard drug (Furosemide).In test small dose group, the serum calcium levels were 16.72 mg% ,which were slightly higher than that of normal and standard groups but much lower than that of the controlled group, hence the given herbal extract showed Antiurolithic activity when administered prior to the feed as it prevented stone formation. In the test large dose, the serum calcium levels were 16.007 mg% which was in similar lines with that of standard and normal levels. Hence here we can conclude that Antiurolithic activity of Syzygium cumini dose dependant, higher dose showed more reduction in serum calcium levels. Similar kind of analysis can be applied for other serum levels of the given components. Hence Syzygium cumini is shows a significant Antiurolithic activity. Similarly, if the kidney weights of the rats when considered, in the normal group the kidney weights ranges between 0.345 – 0.348 gms which is at normal levels because the stones were not induced in them. But in case of controlled group, the kidney weight ranges between 0.745 – 0.758 Gms, this abnormal increase in weight is due to induction of stone through feed. In case of standard group, the kidney weights were maintained at normal levels of 0.445 – 0.465 gms due to the Antiurolithic activity of the standard drug (Furosemide).In case of test small dose group, the kidney weights were maintained under 0.463 – 0.505 gms which are close to the normal levels which shows that the herbal drug (Syzygium cumini) has Antiurolithic activity. In case of test large dose, the kidney weights were maintained under 0.470 – 0.491gms, which are little bit lower than that of the test small dose group. Hence it can be concluded that the Antiurolithic activity of the herbal extract is dose dependant.

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