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An Overview Of The In- Vivo And In- Vitro Models Used To Assess Potent Antiurolithiatic Drugs

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Abstract.
The development of kidney stones, known as urolithiasis, involves complex physicochemical processes, including, nucleation, growth, aggregation, supersaturation and retention in the kidney. While Extracorporeal Shock Wave Lithotripsy (ESWL) is a commonly employed procedure to treating kidney stones, it carries the risk of stone recurrence and infection, and exposure to therapeutic shock waves can lead to acute renal damage. Despite advancements in understanding the biology and symptoms of kidney stones, effective medications for clinical therapy remain elusive. As an alternative or complementary approach, phototherapeutic drugs from medicinal herbs have shown promise in both in vitro and vivo studies for the treatment of lithiasis. These phytomedicine remedies are considered safe, effective, socially acceptable, and often have fewer side effects compared to synthetic medications, making them a traditional choice for kidney stone treatment throughout history. Through a comprehensive evaluation, various single herbs have been tested in animal models of urolithiasis. Results from these studies have demonstrated that plant extracts effectively reduce levels of oxalate, calcium, creatinine, phosphate, uric acid, and BUN, thereby preventing kidney stone formation. This review paper provides a comprehensive overview of different animal models used for evaluating antiurolithiatic activity .In this review, all screening models are visible.
Keywords-*Animal model, Calcium oxalate, Methodology, Urolithiasis*

Introduction

Nephrolithiasis, also known as kidney stone disease, is a condition that is characterized by the formation and retention of crystalline mineral deposits in the renal system. This condition is prevalent all over the world and occurs frequently [1]. There is a considerable clinical and economic burden associated with it, as it affects around 10–15% of the global population and is exhibiting a rising incidence in both industrialized and developing countries as a result of changes in food patterns, lifestyle, and climate [2]. The pathogenesis of kidney stones is extremely complicated, as it involves intricate interactions between metabolic, genetic, and environmental factors [3]. These interactions culminate in the supersaturation of urine with stone-forming salts, nucleation, crystal growth, aggregation, adhesion, and retention within renal tubules or papillary tissue [4]. Despite the fact that calcium oxalate makes up the vast majority of calculi, stones may also contain uric acid, cystine, or chemicals connected to infections [5]. The presence of hypercalciuria, hyperoxaluria, hypocitraturia, changed urine pH, dehydration, and abnormalities in intestinal absorption and renal tubular transport are among the most important risk factors [6].
There is a need for a more nuanced understanding of the multifaceted etiology and underlying processes of kidney stone disease in order to develop effective treatment and preventative methods for the condition. Despite the fact that clinical therapies, which can include anything from dietary modifications and medication to minimally invasive surgical techniques, have the potential to diminish recurrence, the relapse rate continues to be significant [7]. Therefore, the identification of more powerful and focused antiurolithiatic drugs is a major scientific necessity. In order to accomplish this goal, preclinical studies are significantly dependent on the utilization of reliable in vivo and in vitro experimental models [8]. These models are utilized to shed light on pathophysiology, screen potential drug candidates, and progress translational research [9]. In vivo models, which typically involve mice, are used to imitate the production of stones through the administration of lithogenic chemicals like ethylene glycol or ammonium chloride, as well as through dietary modifications or genetic mutation [10]. Under physiological conditions that are very similar to those of human renal lithiasis, these animal models are extremely useful for investigating

systemic interactions, disease progression, treatment efficacy, and potential adverse effects [11]. In the process of data interpretation, however, it is necessary to take into account the fact that there are interspecies differences in renal anatomy and metabolism, as well as ethical limits and the artificial character of stone induction [12].

In vitro models are a key component of antiurolithiatic medication research. They provide controlled platforms for the mechanistic dissection of specific processes such as crystal nucleation, growth, aggregation, and dissolution. Animal studies are a complementary component of this study [13]. In order to recreate important renal microenvironments, these models make use of artificial urine, cell cultures, or microfluidic devices. This allows for a rapid and cost-effective evaluation of the influence that particular medicines, ions, proteins, or inhibitors have on each step of stone formation [14]. In vitro methods are particularly well-suited for high-throughput screening as well as for exploring the cellular and molecular interactions that occur between urinary crystals and renal epithelial cells. These interactions include cytotoxicity, oxidative stress, inflammation, and epithelial-mesenchymal transitions [15]. These models, despite the fact that they offer essential mechanistic insights, do not possess the holistic complexity of whole-organism physiology. Furthermore, they frequently fail to reflect systemic reactions, pharmacokinetic properties, or the interaction between immunological, endocrine, and genetic component [16].
The development of more physiologically relevant models, such as organ-on-a-chip technologies, three-dimensional cultures, and genetically engineered animals, which better recapitulate human disease features and promise enhanced translational fidelity, is helping to bridge these gaps [17]. Recent scientific advancements are helping to bridge these gaps. The pipeline for the development and evaluation of novel antiurolithiatic drugs is further enhanced by the use of multi-omics, sophisticated imaging, and computational modeling [18]. It is therefore the cornerstone of contemporary nephrolithiasis research that the synergy between traditional and innovative in vivo as well as in vitro experimental systems forms. This synergy makes it possible to conduct rigorous evaluations of new therapeutics and provides essential insights into the intricate biology that underpins stone disease [19].

1. Pathogenesis of Urolithiasis

Urolithiasis, or kidney stone disease, is a complex medical disorder characterized by the formation and retention of crystalline stones in the urinary tract, predominantly within the kidney. The pathophysiology of urolithiasis is intricate, including a combination of physicochemical, cellular, molecular, genetic, metabolic, and environmental variables, leading to urine supersaturation, crystallization, and subsequent stone formation [20]. The subsequent narrative provides a comprehensive analysis substantiated by current journal literature of the mechanisms that facilitate the formation and maintenance of urinary calculi.

1.1. Urinary Supersaturation: The Physicochemical Basis

The fundamental mechanism of stone formation is urinary supersaturation, characterized by the concentration of stone-forming elements, including calcium, oxalate, phosphate, uric acid, cystine, or struvite, beyond their solubility in urine. Supersaturation conditions are affected by genetic, nutritional, metabolic, and environmental factors, which facilitate the precipitation of solutes into tiny crystals. Primary factors encompass hypercalciuria, hyperoxaluria, hypocitraturia, diminished urine volume (dehydration), modified urinary pH (acidic for uric acid or alkaline for struvite stones), and elevated dietary consumption of animal protein and sodium [21]. Urine contains inhibitors such as citrate, magnesium, nephrocalcin, Tamm-Horsfall protein, and glycosaminoglycans, which typically suppress nucleation, crystal development, and aggregation; deficits in these substances elevate the risk further [22].

1.1.1. Phases of Calculus Formation: From Nucleation to Retention

1.1.1.1. Nucleation

The preliminary phase entails the conversion of dissolved ions into a stable crystalline state. Nucleation can transpire in free solution (homogeneous) or on pre-existing surfaces, including cell membranes, urinary debris, or Randall's plaques (heterogeneous). Diverse promoters (calcium, oxalate, uric acid, etc.) and inhibitors (citrate, glycoproteins) affect this intricate equilibrium [23].

1.1.1.2. Crystal Development and Aggregation

Upon the establishment of nuclei, additional ions are incorporated into the existing crystals, facilitating their development. Crystal crystal aggregation, the process by which smaller crystals coalesce into bigger clusters, is equally significant; only larger aggregates are likely to be maintained within the kidney, hence enhancing the likelihood of clinical stone development instead of being eliminated in urine [24].

1.1.1.3. Crystal Retention and Adhesion

For a stone to develop, crystals must attach to the renal tubular epithelium or papillary surfaces rather than being ejected. Retention sites encompass regions with anatomical blockage, tubular injury, or papillary tissue revealed by Randall's plaque. Randall's plaques are subepithelial accumulations of hydroxyapatite that act as a nucleus for calcium oxalate stones, especially in individuals with idiopathic calcium stone formation. Cellular damage, frequently resulting from oxidative stress or inflammation, reveals adhesion molecules that facilitate crystal attachment [25].

1.1.1.4. Stone Maturation and Development

The prolonged deposition of additional crystalline material and organic matrix results in stone growth. The organic matrix, consisting of proteins, lipids, and polysaccharides, binds and stabilizes developing stones, influences mineral composition, and enhances stone durability [26].

1.2. Cellular and Molecular Mechanisms

Renal tubular epithelial cells actively engage in processes such as apoptosis, autophagy, or inflammation upon interaction with crystals, hence promoting more crystal retention. Adhesion molecules, including osteopontin, annexin II, hyaluronic acid, and glycosaminoglycans, are upregulated in response to crystal exposure and facilitate cell-crystal interactions, frequently via intricate signaling pathways that involve oxidative stress and the mitochondrial permeability transition pore [27].

Oxidative stress has become a significant mechanistic pathway, wherein the interaction between crystals and tubular cells leads to mitochondrial malfunction, the generation of reactive oxygen species (ROS), and resultant cellular harm. Injured cells reveal adhesion molecules and secrete inflammatory mediators, hence exacerbating crystal adherence and stone proliferation [28].

1.3. Inflammation and Immune Responses

The immune system, especially macrophages, influences the outcome of intratubular or interstitial crystals. Macrophages manifest in two primary phenotypes: pro-inflammatory (M1) and anti-inflammatory (M2). M1 macrophages facilitate stone formation by exacerbating inflammation, but M2 macrophages can phagocytose and eliminate crystals, thereby restricting disease progression. Signaling via pathways such as the NLRP3 inflammasome is crucial: knockout experiments in mice demonstrate decreased stone formation upon NLRP3 inhibition, corroborating its involvement in promoting stone formation. Moreover, inflammatory cytokines (TNF-alpha, IL-1β) and elevated reactive oxygen species compromise epithelial integrity and promote crystal retention, establishing a detrimental loop of damage and urolithiasis [29].

1.4. Function of the Microbiome

Specific bacteria, particularly urease-producing species such as Proteus mirabilis, Klebsiella, and Pseudomonas, are implicated in the formation of infection-related (struvite) stones. Urease hydrolyzes urea into ammonia, increasing urine pH and resulting in the precipitation of magnesium ammonium phosphate (struvite) and/or carbonate apatite calculi. The evolving significance of the extensive urine and gut microbiome is currently being examined, as microbiota might affect urinary composition, immunological responses, and mucosal health [30,31,32].

1.5. Genetic and Systemic Influences

Genetic propensity is well-documented, characterized by familial clustering and recognized mutations in genes like CLDN14, NPT2, calcium-sensing receptor, and vitamin D receptor, among others. Single nucleotide polymorphisms and genome-wide association studies across varied populations have associated these genetic variants with renal calcium and phosphate regulation, supporting a polygenic risk model. Moreover, systemic illnesses such as metabolic syndrome, diabetes, gout, bowel ailments, and specific inherited metabolic disorders serve as direct or indirect risk factors by modifying urine solute equilibrium or pH levels [33,34].

1.6. Pharmacologically Induced and Secondary Calculi

Specific medications (e.g., topiramate, atazanavir, protease inhibitors) and excessive vitamin C may elevate the risk of stone formation by altering urine chemistry or serving as substrates for crystallization. Other medical conditions hyperparathyroidism, distal renal tubular acidosis, or chronic urinary tract infection can alter the risk profile for specific types of stones [35,36,37].

Table 1. Key Stages and Factors in Urolithiasis Pathophysiology

Stage	Description	Key Factors and Mechanisms
Urinary Supersaturation	Excess of stone-forming solutes in urine	Hypercalciuria, hyperoxaluria, uric acid, low urine volume, low citrate
Nucleation	Formation of stable crystalline nucleus	Urine chemistry, inhibitors/promoters, seed surfaces
Crystal Growth & Aggregation	Enlargement and clustering of crystals	Further supersaturation, lack of inhibitors, aggregation
Crystal Retention & Adhesion	Adherence to renal tissues, papillae	Cell injury, Randall's plaques, adhesion molecules, ROS, inflammation
Stone Maturation	Further mineral and matrix deposition	Ongoing supersaturation, organic matrix incorporation

Immunoinflammatory Modulation	Local immune cell response to crystals	Macrophage polarization, NLRP3 inflammasome, cytokines
Stage	Description	Key Factors and Mechanisms
Microbiome & Infection	Bacterial involvement in struvite & carbonate apatite stones	Urease activity, altered urine pH, chronic UTI
Genetic and Systemic Disorders	Underlying hereditary or acquired metabolic diseases	Genetic mutations, diabetes, metabolic syndrome
Drug-Induced Mechanisms	Secondary to altered urine chemistry by medications	Protease inhibitors, anticonvulsants, diuretics, vitamin excess

1.7. Other Risk Factors: Several lifestyle and dietary factors can also contribute to urolithiasis, including a diet high in oxalate or calcium, high-protein diets, excessive salt intake, and certain medical conditions like hyperparathyroidism and inflammatory bowel disease. As the stones grow in size, they can obstruct the urinary tract, causing symptoms like severe pain (renal colic), hematuria (blood in urine), frequent urination, and urinary tract infections. Treatment options for urolithiasis depend on the size, location, and composition of the stones and may include pain management, hydration, medications, or surgical interventions to remove or break up the stones.

The chemical makeup of kidney stones is used to categorise them. For crystals to develop, the urine needs to be in a state of supersaturation compared to the stone's composition. This implies that the concentrations of substances must surpass the thermodynamic solubility of the material.

This critical factor is closely linked to the specific types of stones that come into being [38]. Lowering urine supersaturation helps prevent stone recurrence. More than 80% of all stones are composed of CaOx [39], the most common stone component. Along with various kinds of stones, the remaining 20% is made up of struvite, cystine, and uric acid [40]. Disorders classified as metabolic include hypercalciuria (excretion of calcium in the urine over 200 mg/d), hypocitraturia (excretion of citrate in the urine less than 320 mg/d), and hyperoxaluria (excretion of oxalate in the urine over 40 mg/d). Furthermore, gout is classified as a metabolic illness and is defined by excretion of excessive amounts of uric acid (600 mg or more) and insufficient amounts of magnesium (less than 50 mg/day) [41]. The supersaturation and crystallization of CaOx in the kidney could be affected by any cellular abnormality that might affect different urine ions and other chemicals. Calcium phosphate (CaP) and calcium oxalate (CaOx) crystals are prevalent in the urinary system. Even though humans eliminate countless urine crystals daily, preventing their transformation into kidney stones, this process indicates that the state of supersaturation is transient and not constant [41,42]. The creation of stones Crystals must be kept within the kidney and placed where they can create a stone nidus by ulcerating and moving to the renal papillary surface [43]. Because of plants, which are viewed as being quiet, secure, safe, having few or adverse effects, cost effective, conveniently available, and economical, the majority of people in the world still have access to affordable medications. Stone disease is often addressed through surveillance and surgical stone removal. From observation to surgical stone removal, stone disease is generally

managed. Several variables, including stone size, symptom severity, obstruction level, kidney function, stone location, and the presence of infection, can impact the choice of intervention [44]. We investigated the efficacy of individual plant extracts as potential preventive or therapeutic agents for renal stones in a rat model. In order to gather significant information for comprehending the mechanisms of medications with possible antiurolithiatic effects, our goal was to thoroughly evaluate *in-vivo, in-vitro*, and surgical urolithiasis models. Several therapeutic plants were examined by *in-vivo, in-vitro*, and surgical techniques in order to determine their possible antiurolithiatic effects. Numerous *in-vivo* and *invitro* models have been developed to investigate the fundamental mechanisms of urinary stone formation and assess the effects of various treatment modalities on the onset and development of the disease. Rats are the most common animal used in models to research kidney stone development because the build-up of calcium oxalate (CaOx) in the kidneys mimics the cause of kidney stones in people [45].

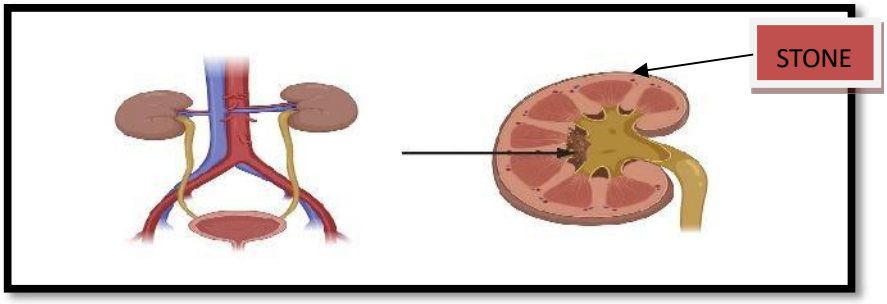

Fig. 1. Urolithiasis

2. IN VIVO MODEL

When animals are provided with drinking water containing 25-10% of oxalate, they experience the formation of oxalate stones in their urinary tracts. Oxalic acid, a breakdown product of Ethane-1,2-diol, can crystallize as calcium oxalate within the body's tissues when present in sufficient quantities. Hyperoxaluria, a disorder that increases oxalate retention and excretion in the kidneys, aids in the development of kidney stones.

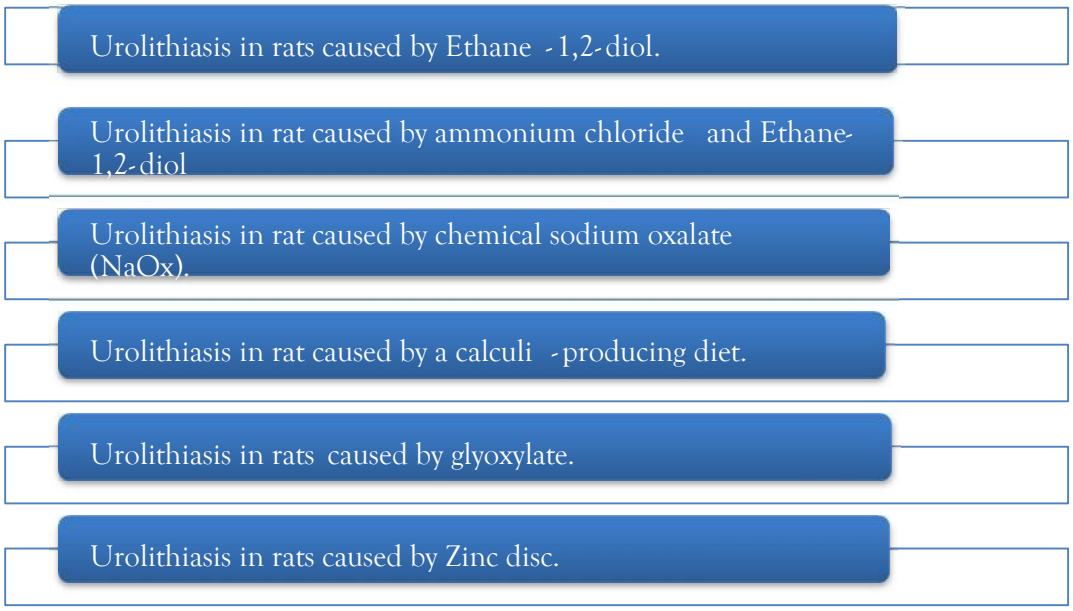

Fig. 2. Types of *in-vivo* animal model

Stone formation is more significantly impacted by variations in urine oxalate levels than calcium levels. It is well known that higher urinary calcium favors the nucleation, precipitation, and crystallization of calcium oxalate or apatite (calcium phosphate) in urine. When paired with oxalate stress, the presence of calcium phosphate crystals can promote calcium oxalate deposition and foster the production of stones. The urinary tract stone disease known as urolithiasis can cause a decrease in the glomerular filtration rate (GFR). Waste products accumulate in the blood as a result of this decline in GFR, especially nitrogenous compounds including urea, creatinine, and uric acid. Magnesium is known for preventing urine crystallization. Magnesium's significance in preventing stone formation is supported by the fact that those who produce stones frequently have low urine magnesium contents. Additionally, it has been noted that magnesium slows the pace at which calcium oxalate crystals form. Overall, factors like urinary calcium and magnesium concentrations and oxalate concentrations in drinking water can have a big impact on kidney function and the development of kidney stones [46,47].

2.1. Urolithiasis in rats caused by Ethane-1,2-diol Model

The primary cause of the oxidative damage must be attributed to the high concentration of oxalate Ethane-1,2-diol (EG) produces, which accounts for 0.75 percent of the lithogenic activity. The EG rat model must therefore be considered an appealing model to assess the creation of renal papillary stones, at least for those stones whose origin is related to oxidative cell damage, even though it can be questioned as a general paradigm to research renal stone formation. To encourage CaOx deposition and hyperoxaluria, the kidneys are administered 2% ammonium chloride (w/v).

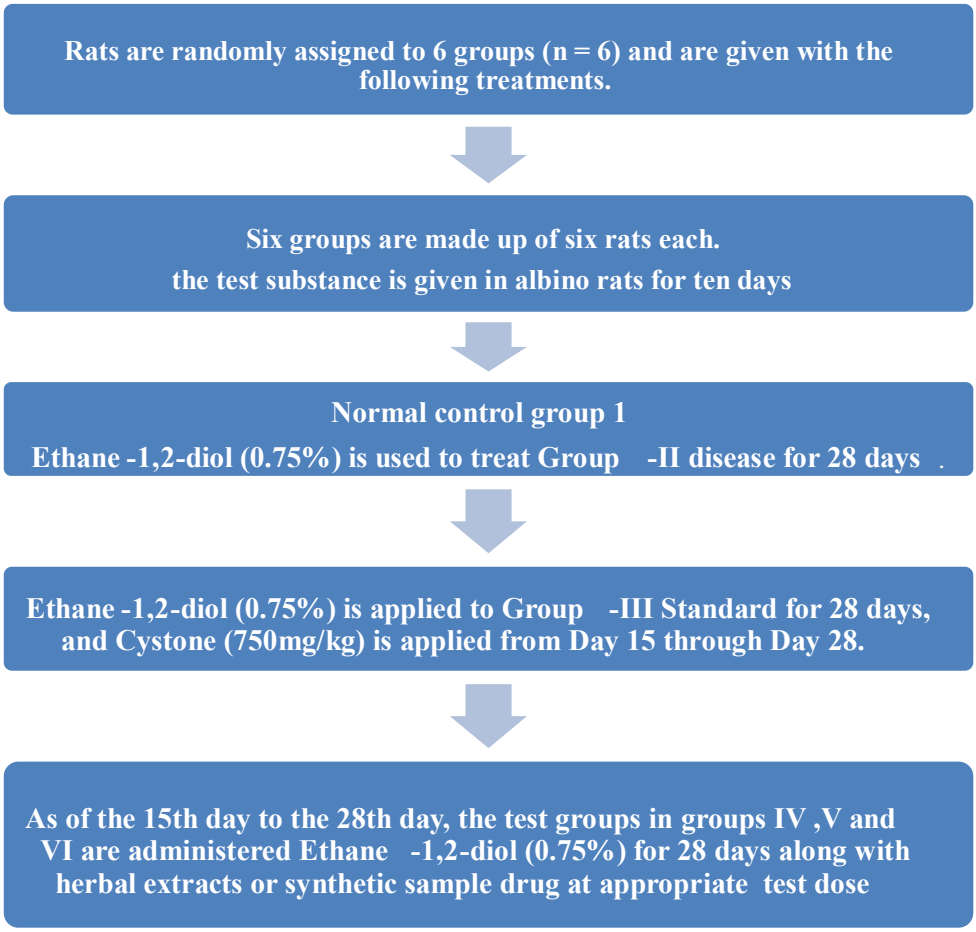

Figure: 3 Urolithiasis in rats caused by Ethane-1,2-diol

2.2. Ethane-1,2-diol and ammonium chloride induced urolithiasis model

Fig.3. Ethane-1,2-diol and ammonium chloride induced urolithiasis model Throughout the study, the rat's weights are measured daily to monitor any potential changes [48].

2.3. Urolithiasis in rat caused by chemical sodium oxalate (NaOx)

Intraperitoneal injection of sodium oxalate is used to induce oxalo calcic lithiasis (NaOx). The observed weight loss is attributed to anorexia triggered by disruptions in the metabolism of proteins, carbohydrates, or fats caused by sodium oxalate injection. The harmful effects are associated with the formation of insoluble CaOx salts, leading to CaOx nephrolithiasis at physiological pH. Time passed between the initial NaOx injection and subsequent injections appears to affect the degree of necrosis, the size of CaOx particles, their amount, and their distribution inside the inner medulla [49,50].

2.4. Urolithiasis in rat caused by a calculi-producing diet

Glycolic acid ingestion causes glycolate oxidase, a liver enzyme involved in the production of oxalate, to increase. Calcium oxalate (CaOx) stones can develop when a regular diet is combined with 3 percent glycolic acid. This combination of glycolic acid and the typical diet causes calcium and oxalate to build up within the kidney tissue over the course of 40 days. The development of papillary calcification and the eventual production of calculi in the renal tissue are linked to the elevated levels of calcium and oxalate. To induce hyperoxaluria and calcium oxalate deposition in the kidney (referred to as CPD), a method involving the administration of gentamycin (at a dose of 40 mg/kg/day subcutaneously) and a calculi-producing diet was employed. The latter involved combining the standard rat pellet feed with 5 percent ammonium oxalate, pelletizing the mixture, and subsequently drying it [51].

2.5. Glyoxylate induced acute lithiasis

An acute glyoxylate poisoning that significantly increases the calcium, oxalate, and phosphorus deposits in the kidneys can result in glyoxylic lithiasis. The amount of oxalate in the urine quadruples, has been reported. In tests using isolated rat hepatocytes, glycollate and glyoxylate effectively function as oxalate precursors. The only chemical proven to be an instant replacement for oxalate in humans is glyoxylate. Glyoxylate offers two advantages

1. The capacity to accurately regulate the amount of harmful material absorbed.
2. It facilitates the investigation of real antilithiatic chemicals by acting as a direct precursor tooxalic acid.

To induce oxalate stones in animals within 24 hours, sodium glyoxylate was administered intraperitoneally at a dose of 120 mg/kg per rat, administered in two consecutive doses in the morning and evening [52].

2.6. Urolithiasis in rats brought on by a zinc disc

Rats are put to sleep with an Intraperitoneal injection of sodium pentobarbital (40 mg/kg). A suprapubic incision makes it possible to see the urinary bladder, and a small cut is made near the bladder's apex. After the urine's pH is assessed using narrow range pH paper (BDH), a sterile vial is used to collect it. The rats have sterile zinc discs inserted into their bladders, followed by a week of rest. The wound is closed with one stitch of absorbable 4-0 chronic catgut (Ethicon). When zinc foreign entities is implanted in the pee bladder, the smooth muscle of the organ expanded larger and urine stones developed as a result [53,54,55].

3. In-Vitro Studies of Antiurolithic Study

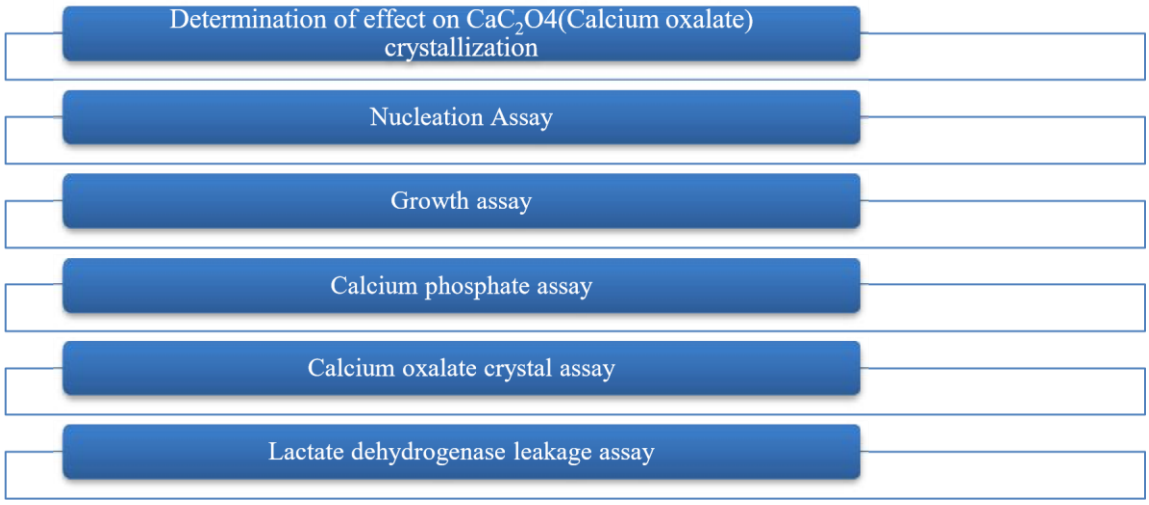

Fig. 5. *In-vitro* studies of antiurolithic study

3.1. In vitro calcium oxalate crystallization assays (Nucleation assay)

To assess the impact of test compounds on the nucleation, growth, and shape of calcium oxalate crystals. Formulate solutions of calcium chloride ($CaCl_2$) and sodium oxalate ($Na_2C_2O_4$) in deionized water or synthetic urine at specified quantities (often 4–10 mmol/L

$CaCl_2$ and 50 mmol/L $Na_2C_2O_4$). Modify the pH to physiological values (about 5.5 to 6.5) via HCl or NaOH. Combine equal volumes of calcium chloride and sodium oxalate solutions swiftly while maintaining continuous agitation at room temperature or 37 °C for a specified duration (e.g., 20–60 minutes). The test substance (drug, plant extract) is incorporated before to or during mixing to examine its influence on crystallization. During crystallization, measure optical density or turbidity at 620 nm with a spectrophotometer to observe crystal nucleation and growth dynamics. Ultimately, crystals can be harvested using filtration, desiccated (e.g., at 80 °C for 1 hour), and examined via microscopy, Raman spectroscopy, or X-ray diffraction for evaluation of size, shape, and composition. The percentage inhibition of nucleation or growth is determined by comparing the absorbance or crystal characteristics of test samples with those of the control (without inhibitor) [56, 57].

3.2. Crystal Aggregation Assays (Growth assay)

Stone slurry is prepared at a concentration of 1.5 mg/ml in a sodium acetate buffer with a pH 5.7. A test solution containing 10 mM Tris-HCl and 90 mM NaCl is adjusted to a pH 7.2 using a solution-depletion test with a seed. In this experiment, a solution containing 1 mM $CaCl_2$ and 1 mM sodium oxalate ($Na_2C_2O_2$) is introduced to a crystal seed of CaOx monohydrate. The reaction between $CaCl_2$ and $Na_2C_2O_2$ on the crystal seed led to the accumulation of CaOx (CaC_2O_4) on the crystal surfaces. This accumulation caused a reduction in the detectable free oxalate concentration, as measured by a spectrophotometer at 214 nm. When the test sample is added to this solution, the depletion of free oxalate ions is affected. If the test sample inhibited the growth of CaOx crystals, the amount of free oxalate ions depleted would decrease. The extent of this effect was determined by calculating the rate of reduction in free oxalate ions. This calculation involved comparing the initial rate to the rate observed

after a 30-second incubation, both with and without the test sample [58,59]. To quantify the relative inhibitory activity of the test sample, a percentage was calculated using the formula.

Relative Inhibitory Activity=(C−S/C) ×100

Here, C represents the rate of free oxalate reduction without the test sample, and S represents the rate of free oxalate reduction with the test sample. This formula allows for the assessment of the test sample's ability to inhibit the growth of CaOx crystals, indicating its inhibitory potential as a percentage

3.3. Crystal Dissolution Assays

The efficacy of substances in dissolving or degrading premade calcium oxalate crystals, simulating therapeutic outcomes is assessed. In this model, Pre-synthesize calcium oxalate crystals as outlined, then collect and weigh a specified quantity. Disperse crystals in a physiological buffer or synthetic urine medium, with or without test chemicals. Incubate the suspension at 37 °C for specified time intervals (e.g., hours to days) with constant agitation. At regular intervals, aliquots are extracted, filtered, and the quantity of dissolved calcium or oxalate ions in the supernatant is quantified, often by titrimetric methods employing potassium permanganate or spectrophotometric techniques. Determine the percentage of crystal dissolution induced by the test agent in relation to the control group [60,61].

3.4. Inhibition Assay

Fifty milliliters of mixed extract are placed in a beaker, and two salt-forming solutions were added dropwise using burettes to create the reservoir. Subsequently, the mixture is subjected to boiling for 10 minutes on a heating mantle, followed by cooling to ambient temperature. The precipitate is obtained by centrifuging small quantities sequentially, while the supernatant was dumped into a pre-weighed centrifuge tube. Subsequent to drying in a hot air oven, the tube containing the precipitate was allowed to cool to room temperature and weighed using a balance until a steady weight is attained. The weight of the precipitate is determined. Water serves as the control in this experiment. All tests are conducted at ambient temperature. The subsequent formula is employed to determine the % efficiency of both individual and mixed extracts [62].

$$Percentage\ inhibition = \frac{Wb - Ws}{Wb} x100$$

3.5. Egg Membrane Preparation Method

3.5.1. Procurement and Decalcification of Eggshell

Obtain fresh chicken eggs from local grocery or farm. Remove the outer calcified shell by immersing the eggs in concentrated hydrochloric acid (HCl, about 2N or concentrated) for 24 hours at room temperature. This process completely decalcifies the shell, leaving the
inner egg membrane intact. After decalcification, carefully remove the softened shell remnants, leaving only the semi-permeable egg membrane.

3.5.2. Cleaning and Membrane Handling:

Wash the decalcified egg membranes thoroughly with distilled water multiple times to remove any residual acid and extraneous material. Using a sharp pointer, make a small hole at the top of the

membrane and squeeze out the egg contents completely.Rinse the membrane again with distilled water. Place the membranes in an ammonia solution briefly while moist, to neutralize and further clean, then rinse again with distilled water. Store the cleaned membranes refrigerated in a moist condition at pH 7 to 7.4 until use.

3.5.3. Calcium Oxalate Crystal Preparation 3.5.4. Assembly of Egg Membrane Model

Take a defined amount of prepared calcium oxalate crystals (e.g., 10 mg) and the test compound or plant extract (e.g., 0.5 ml aqueous extract). Pack these materials carefully inside the prepared semi-permeable egg membrane. The membrane is securely sutured or sealed to prevent leakage. Suspend the membrane inside a conical flask containing 100 ml of a 0.1 M TRIS buffer at pH around 7.4.

3.5.5. Incubation and Measurement

Incubate the flask at 37 °C in an incubator for a predetermined period (commonly 2 hours). After incubation, retrieve the egg membrane, and the contents are collected into test tubes.

To each tube, add 2 ml of 1N sulfuric acid, then titrate with potassium permanganate (KMnO4, standardized) until a light pink endpoint is achieved. The amount of KMnO4 used correlates to the calcium content, serving as a quantitative measure of calcium oxalate crystals dissolved or retained. Calculate the percentage dissolution or inhibition by comparing experimental groups with controls [63,64].

3.6. Calcium phosphate assay

This involves the study of calcium phosphate (CaP) formation, growth, and demineralization in *in vitro* homogeneous systems. The experimental procedure:

3.6.1. $CaPO_4$ formation system preparation

A 5.0 ml system is made by mixing 0.5 ml of KH_2PO_4 (50 mM), 0.5 ml of $CaCl_2$, and 2.5 ml of Tris buffer (210 mM NaCl + 0.1 mM Tris HCl).

To keep the overall volume at 5.0 ml, the volume of the test material is adjusted from 0.2 ml to 1.5 ml, while the volume of water is decreased from 1.5 ml to 0.0 ml. Following a 4500rpm centrifugation of the system, the precipitates are collected and dissolved in 5 ml of 0.1 N HCl.

3.6.2. **$CaPO_4$ Growth:**

A second batch of 5.0 ml systems is made according to established procedures. In the same tubes, these systems are grown again with increasing amounts of the test drug. The precipitates' calcium and phosphate contents is calculated and dissolved in 0.1 N HCl.

3.6.3. Demineralization Test:

A 5.0 ml system is created for the demineralization test without the addition of any test substance (control group). The control group's precipitates are gathered. To these precipitates, Tris buffer (210 mM NaCl + 0.1 mM Tris HCl) is added, along with different amounts of the test material (from 0.2 ml to 1.5 ml), while the amount of water is decreased. The system is centrifuged for 15 minutes at 4500 rpm to measure the amounts of calcium and phosphate in the supernatant that was left over [65,66].

3.6.4. **Calcium and Phosphate Ion Estimation**:

Using particular techniques, calcium and phosphate ions in the precipitates and supernatant were determined [67,68].

3.6.5. Percentage Inhibition Calculation:

The formula used to determine the percentage inhibition of the mineral phase when the test substance is present is (C - T) / C) x 100 = % inhibition, where C is the amount of Ca^{2+} or HPO_4^{2-} ions in the precipitate that forms in the control system with distilled water and no test substance, and T is the amount of Ca^{2+} or HPO_4^{2-} ions in the precipitates that formed in the test system with the test substance.

4. Limitations and Future Scope

In vitro models for kidney stone research provide meticulous regulation of experimental parameters, including crystallization, nucleation, aggregation, and dissolution, yielding significant insights into the initial mechanistic stages of stone development. Nonetheless, these models intrinsically lack the physiological intricacy of the renal milieu. They do not emulate dynamic elements such as urine flow, renal epithelial contacts, immune system participation, or the organic matrix including proteins present in natural stones, which can substantially affect crystal behavior [69]. Moreover, numerous in vitro research employ simplified and non-physiological circumstances concerning ion concentrations, pH, and supersaturation, hence constraining their direct relevance to human kidney stone etiology. They are also unable to consider systemic mechanisms like as metabolism, hormone control, and pharmacokinetics that influence stone formation and therapeutic efficacy [70].

In vivo models, generally utilizing rodents or other animals subjected to chemical inducers such as ethylene glycol or ammonium chloride, endeavor to replicate the multifaceted biological context of nephrolithiasis, encompassing stone formation, growth, retention, and passage within the framework of renal physiology and immune responses. Nonetheless, these models exhibit numerous limitations: variations in renal anatomy, metabolism, and stone composition among species may diminish their applicability to human pathology; stone formation is frequently artificially induced and may not accurately reflect natural disease progression; biological variability across strains and conditions impacts reproducibility; and ethical and financial constraints restrict the scope and scale of studies. Furthermore, pinpointing particular mechanistic pathways in vivo is difficult due to systemic intricacy [71]. Anticipating the future, technological advancements are expected to mitigate these constraints. Improving in vitro models by the integration of kidney cell cultures, threedimensional extracellular matrices, and microfluidic "organ-on-a-chip" devices can more accurately replicate renal microenvironments, encompassing fluid dynamics and cell–crystal interactions, hence augmenting physiological relevance [72]. The integration of multi-omics and systems biology methodologies with experimental models helps enhance comprehension of the many biological mechanisms underlying urolithiasis. Genetically modified mice that closely mimic human metabolic and genetic differences related to stone disease are emerging in animal models, facilitating detailed research of etiology [73]. Furthermore, contemporary computer modeling and machine learning applications in imaging and clinical data analysis are enhancing kidney stone detection, risk assessment, and individualized treatment options, potentially diminishing reliance on animal models [74]. Ethical and economical methodologies, including 3D bioprinting and in silico simulations, present promising prospects for future study [75]. The ongoing enhancement, standardization, and integration of these complimentary approaches will be essential for advancing translational accuracy in the development of antiurolithiatic drugs and the management of kidney stone disease [76]. Consequently, although existing *in vitro* and *in vivo* models have proven essential for kidney stone research, their limitations highlight the necessity for new, integrated, and physiologically pertinent systems that might expedite the identification and clinical application of more effective medicines [77].

Conclusion

A variety of in vitro and in vivo models have been crucial in enhancing our comprehension of kidney stone development and in assessing antiurolithiatic treatments. In vitro models such as tests for crystallization, nucleation, aggregation, and dissolution facilitate regulated and quick screening of

processes by clarifying how medicines or extracts affect crystal formation and growth under specified biochemical circumstances. These models are especially useful for analyzing the contributions of urinary supersaturation, crystal inhibitors, pH, and certain ion concentrations in the initial phases of stone formation. While economical and easily replicable, they fundamentally lack the intricacy of whole-organism physiology. In vivo models, especially those utilizing rodents subjected to substances such as ethylene glycol, ammonium chloride, or diets that induce hyperoxaluria, replicate the complex and dynamic milieu of the mammalian kidney. These animal models enable the evaluation of stone formation, development, retention, and passage within the framework of real-time renal physiology, immunological response, and metabolic interactions. They are essential for assessing medication pharmacodynamics, safety profiles, and systemic effects that cannot be measured in vitro. Nonetheless, interspecies variations and ethical dilemmas provide persistent obstacles. The prudent application of both model systems is essential to close the translational gap in antiurolithiatic drug discovery. In vitro models provide mechanistic insight and high-throughput capabilities for initial screening, but in vivo models deliver the biological relevance essential for predicting treatment efficacy and side effects in humans. The integration of data from various alternative models boosts experimental validity, informs rational medication design, and improves the likelihood of successful clinical translation. As our comprehension of kidney stone formation advances including novel aspects such as the influence of the renal microbiome and immune responses ongoing enhancement and innovation in experimental models are crucial for the advancement of next-generation antiurolithiatic medicines.

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