IN-VITRO
CRYSTAL GROWTH
3.1 INTRODUCTION

Urinary stone disease (urolithiasis) is common worldwide and calcium oxalate is the principal crystalline component in approximately 75% of all renal stones. Most urinary stones probably begin as minute aggregates in the small collecting ducts of the kidney. They then migrate to the pelvicalyceal system, where they increase in size and may then move into the ureters and bladder. The earliest evidence of the disorder is the stones found in mummies\(^1\) entombed in the predynastic Egyptian era, around 4000 BC. The general factors responsible for urinary stone formation are, environmental, genetic, the concentration of the relevant ionic species in the urine, the availability and chemical nature of the macromolecular components of the stone matrix and the concentration of urinary physiological inhibitors of crystallization. Kidney stones are more common in the industrially developed nations and less so in countries whose economies are more dependent on agriculture.

The types of urinary stones are calcium oxalate (the commonest), calcium phosphate, magnesium–ammonium–phosphate, uric acid, sodium and ammonium urates, cystine, xanthine etc. Stones may also be of mixed composition. Deposition of calcium oxalate microcrystal in human body can be a significant problem and it is recognised that 70 – 80% of kidney stones contain calcium oxalate\(^2\). Deposition of calcium oxalate microcrystal in soft tissues has been observed in primary hyper oxaluria, chronic renal failure and certain small bowel diseases\(^3,4\).
The kidneys filter waste products from the blood and add them to the urine that the kidneys produce. When waste materials in the urine do not dissolve completely, crystals and kidney stones are likely to form. Small stones can cause some discomfort as they pass out of the body. Regardless of size, stones may pass out of the kidney, become lodged in the tube that carries urine from the kidney to the bladder (ureter), and cause severe pain that begins in the lower back and radiates to the side or groin. A lodged stone can block the flow of urine, causing pressure to build in the affected ureter and kidney. Increased pressure results in stretching and spasm, which cause severe pain.

3.1.1 Epidemiological factors in the formation of urinary stones.

Epidemiological factors important in the formation of urinary stones are age, race, sex, geographic location, environmental and life factors, diet, fluid intake, nutritional habits and anatomical abnormalities of urinary tract.

3.1.1.1 Prevalence

The prevalence of urinary stones in developed countries ranges from 4 to 15% of the adult males\(^{5-8}\). The stone incidence in females is half as common.

Medical care for each stone incident costs approximately $2,000 in the United States. The projected costs of stone treatments of the entire national
population of adult males are estimated to be $315,000,000$ a year\textsuperscript{9}. The percentage of population which had the incidence of stones in 1980, in USA, Sweden, Germany, Italy and UK was found to be $12.0$, $9.5$, $6.8$, $3.1$ and $1.5$ respectively\textsuperscript{10}.

Incidence of calcium-containing urinary stones in the upper urinary tract has been increasing\textsuperscript{11} in Japan since the Second World War with increasing westernization of life–style and industrialization, with the annual incidence of urolithiasis steadily increasing from $53.8/100,000$ general population in 1965 to $92.5/100,000$ in 1985.

While kidney stone disease is prevalent in all industrialized nations, Louisiana and the Gulf States comprise what is known as the “Stone Belt” where kidney stones occur\textsuperscript{12} at a rate of approximately $5\%$.

In Europe, Africa and Middle East there is prevalence of endemic bladder stones. India, Thailand and Turkey are also countries noted for endemic bladder stones\textsuperscript{13}. High incidence of urolithiasis prevails in north and north western regions of India\textsuperscript{14}. The incidence of urinary stones in various states of India varies between $0.02$ – $2.11\%$. Epidemic nature of the disease has been proved in Punjab, Delhi, Manipur and Rajasthan.

3.1.1.2 Season

The incidence of upper urinary tract stones increases with ambient temperature, mainly due to reduced urine volume. Seasonal differences in
the incidence of urolithiasis in temperate climates are attributed mainly to this mechanism, although seasonal dietary variation (calcium and oxalate intakes) and increased vitamin D synthesis in the skin due to increased exposure to sunlight are also factors. Parry and Lister reported\textsuperscript{15} that increased mean temperature, increased perspirations, slow winds and low rain fall could influence the development of stones in stone prone patients. Stone patients pass more stones in summer than in winter.

3.1.1.3 Sex

Although stones generally occur more frequently in men than in women\textsuperscript{1} (Male: Female ratio about 2.5:1), recent studies in the United Kingdom and Portugal have shown that, within the past 25 years, there has been a progressive decrease in the age at onset of stone formation in both men and women, particularly women.

Within the population of stone formers as a whole, the male : female ratio is now 1.7:1 among patients who formed their first stone before the age of 20 years; but in patients currently aged less than 20 years, the ratio has fallen to 1.1:1. These changes have been attributed to alterations in diet and life style over the last 25 years. In western countries the ratio of male to female with respect to stone formation is 2:1 while in India\textsuperscript{13} it is 5.5:1 (adults) and 7.8:1 (children). The higher incidence of urolithiasis in men than in women is due to their higher excretion rates of oxalate, calcium, uric acid and generally lower citrate excretion, as well as the anatomical differences.
3.1.1.4 Age

Urolithiasis is rare in childhood in the absence of either underlying structural or functional urinary tract abnormalities, or one of the inherited metabolic causes. The lower incidence of calcium oxalate lithiasis in children may be attributed, among other factors, to the stronger inhibition of calcium oxalate crystal aggregation by pediatric urinary macromolecules, which in turn might be affected by the higher concentration of glycosaminoglycan\textsuperscript{16}. Miyake\textsuperscript{17} et al also reported that the excretion rate of citrate and magnesium in urine from children was much higher than in urine from adults. These two stone inhibitors are very likely to elevate the inhibitory activity of urine from children against calcium oxalate crystal growth and nucleation.

3.1.1.5 Complexion

The occurrence of renal stone in South African Blacks is extremely rare\textsuperscript{18}-\textsuperscript{20}. Immunity to urolithiasis has also been reported for certain other race groups elsewhere in the world. For example, Studies at the Darwin Hospital in Australia showed an absence of renal tract calculi in Aborigines despite the fact that this group constitutes 29% of the population served by that institution\textsuperscript{21}. The significantly low incidence of urinary calculi in the American Negro has been well documented\textsuperscript{22-24}. Eskimos also appear to rarely form stones\textsuperscript{25}. 

Modlin has reported extensively on the chemical composition of urine from black subjects and has suggested that their immunity to urolithiasis is due to their singular and immutable dietary habits which result in a urine the composition of which creates a milieu that is inimical to the formation of crystals. On the other hand, renal stone occurs in the white South African population with the same frequency as that reported in other western communities.

The hypothesis that the lower incidence of stone disease in Blacks may be due to a high Na / Ca ratio. It is suggested that various salts play a role in lowering the stone forming potential of such urines by a competitive substitution mechanism in which lattice calcium is displaced by sodium.

3.1.2 Crystalline components of human urinary calculi

The chemical composition of stones depends on the chemical imbalance in the urine. Although kidney stones are generally composed of calcium oxalate or calcium phosphate, they may also consist of uric acid, magnesium–ammonium phosphate or cystine. Stones develop from a wide variety of metabolic or environmental disturbances, including varying forms of hypercalciuria, hypocitraturia, undue urinary acidity, hyperuricosuria, hyperoxaluria, infection with urease–producing organisms and cystinuria.
3.1.2.1 Calcium stones

Most (80 percent) urinary calculi contain calcium oxalate\(^1\), often on its own, but frequently mixed with calcium phosphate or, occasionally, uric acid. The most common cause of calcium stone production is excess calcium in the urine (hypercalciuria). Excess calcium is normally removed from the blood by the kidneys and excreted in the urine. In hypercalciuria, excess calcium builds up in the kidneys and urine, where it combines with other waste products to form stones. Low levels of citrate, high levels of oxalate and uric acid, and inadequate urinary volume may also cause calcium stone formation.

Calcium stones are composed of calcium that is chemically bound to oxalate (calcium oxalate) or phosphate (calcium phosphate). Of these, calcium oxalate is more common. Calcium phosphate stones typically occur in patients with metabolic or hormonal disorders such as hyper parathyroidism and renal tubular acidosis.

Increased intestinal absorption of calcium (absorptive hypercalciuria), excessive hormone levels (hyper parathyroidism), and renal calcium leak (kidney defect that causes excessive calcium to enter the urine) can cause hypercalciuria. Renal tubular acidosis (inherited condition in which the kidneys are unable to excrete acid) significantly reduces urinary citrate and total acid levels and can lead to stone formation, usually calcium phosphate.
3.1.2.2 Infection stones

So-called 'infection stones' composed of magnesium ammonium phosphate, usually in conjunction with calcium phosphate, are more common in women and now constitute between 4 and 15 percent of stones, depending on the country of origin. They are caused by urinary tract infection with urea-splitting organisms that secrete the enzyme urease. This converts urea to ammonium (NH$_4^+$) and bicarbonate, making the urine more alkaline. As a result, phosphate-containing salts, such as calcium phosphate and magnesium ammonium phosphate, precipitate and increase the risk of stone formation. The relative incidence of infection stones has decreased over the past 25 years in most western countries, presumably as a result of better clinical diagnosis and earlier treatment of urinary tract infections.

3.1.2.3 Uric acid stones

Uric acid is the major end product of purine metabolism. There are three factors that promote the formation of uric acid stones: (i) Low urine volume, (ii) acid urine pH and (iii) high uric acid excretion. For a given diet, these stones are more common in elderly men because of the decline in urine pH with age. Because the pK of uric acid /urate is approximately 5.7, a more acid urine pH favours the less soluble undissociated form of uric acid. Pure uric acid stones are infrequent in most developing countries and are most common in the oil-rich states of the Arabian Gulf, or in countries where there is a cheap local source of meat, fish or poultry protein. Most uric acid stones
are idiopathic; a small number form secondary to some disorder of purine metabolism or to a condition in which there is high tissue turnover (such as tumour necrosis following chemotherapy).

3.1.2.4 Rare stones

In all series of stones analysed, between 1 and 2 percent consist of a range of ‘rare’ constituents derived from either some hereditary or congenital inborn error of metabolism\(^1\) such as cystinuria, xanthinuria or from a prescribed drug or metabolite, which is relatively insoluble in urine. Cystine is an amino acid in protein that does not dissolve well. Some people inherit a rare, congenital condition that results in large amounts of cystine in the urine. This condition (called cystinuria) causes cystine stones\(^{30}\) that are difficult to treat and require life—long therapy. Xanthine stones are formed due to either the inborn error of metabolism called xanthinuria, congenital Xanthine oxidase deficiency or a heavy dosage of the Xanthine oxidase inhibitor allopurinol. Silica calculi occur usually in patients taking aluminium trisilicate for the treatment of peptic ulcers\(^{31-33}\). All stones contain a small percentage by weight of mucoproteinaceous matrix. Some ‘stones’ consist almost entirely of mucoprotein, and usually result from inflammation of the urinary tract in patients whose urine is not sufficiently supersaturated to mineralize the organic matrix.
3.1.2.5 Mixed stones

Stones may also be of mixed composition; calcium oxalate stones commonly contain a minute central core of uric acid on which calcium oxalate has initially grown by epitaxy. Epitaxy is defined as the growth of one crystal on a substrate of another crystal with a near geometrical fit between the respective contact planes which must satisfy the bonding requirements of two crystal structures. Lonsdale had investigated a variety of possible epitaxy of the components of urinary calculi and gallstones.

3.1.3 Causes of urinary stone formation

The "free-particle" and "fixed-particle" theories have been advanced to explain stone formation. The "free-particle" model is that stones are initiated when urine becomes so excessively supersaturated with one of the salts or acids occurring in kidney stones that crystals spontaneously precipitate in urine. If this happens frequently, and if the crystals grow or aggregate sufficiently within the transit time of urine through the kidney, then the risk increases that one of these particles will become trapped at some narrow point along the urinary tract and act as a focus around which a stone can form. The fixed model of stone formation which is currently favoured, requires chemical 'fixation' of a crystal, or aggregate of crystals, to the renal epithelial cell lining. This fixed particle may result from injury to the cell wall and/or from some 'gluing' material present only in the urine of stone formers.
that causes crystals to adhere to these sites and then results in stone formation\textsuperscript{38}.

Both models require urine to be supersaturated to some degree with respect to the stone–forming salt or acid concerned, sufficient to cause crystals to be formed by nucleation that is either homogeneous (spontaneous) or heterogeneous (on a pre–existing nucleus of some foreign material). Vermeulen and Lyon\textsuperscript{39} in 1968 proposed the advanced theory by explaining the different stages such as super saturation, nucleation, growth, aggregation and retention involved in the formation of the stone.

Super saturation leading to nucleation is controlled by the thermodynamic free energy of the solution. The process of nucleation results in a reduction of excess free energy to a more thermodynamically stable environment. Aggregation appears to be the major mechanism for crystal growth\textsuperscript{40}. A final factor that is important in the effective growth of renal calculi is the retention of microcrystals in the urinary tract, possibly correlated with prior injury.

### 3.1.4 Modifiers of crystallization

Urine contains substances (crystallization modifiers) that inhibit the nucleation, growth, aggregation and cell attachment of crystals. These substances may function to protect the kidney against the possibility of pathological calcification in tubular fluid and urine\textsuperscript{41}, which are generally
supersaturated with respect to calcium salts, thereby preventing stone formation. The inhibitors of crystallization include magnesium, citrate, pyrophosphate, ADP, ATP, at least two phosphopeptides, glycosaminoglycans, Tamm–Horsfall protein, nephrocalcin, calgranulin, fibronectin, various plasma proteins, osteopontin (uropontin), α₁ - microglobulin, β₂ - microglobulin, urinary prothrombin fragment 1, and inter-α-trypsin inhibitor. The second group of modifiers is claimed to promote one or more of the processes involved in crystallization. These are known as promoters of stone formation and include, matrix substance A, various uncharacterized urinary proteins and glycoproteins and the polymerized form of Tamm–Horsfall protein (uromucoid).

Tamm–Horsfall glycoprotein (a normal urinary constituent) inhibits calcium oxalate crystal aggregation at high pH values and low ionic strength. Raising the ionic strength and lowering the pH normally reduces its ability to inhibit crystal aggregation. Some specimens of Tamm–Horsfall glycoproteins, particularly those isolated from stone–former’s urine, promote crystal aggregation in the presence of additional calcium ions and have an abnormally high ability to polymerize. An inherited abnormality of this type may be a factor in the aetiology of urinary stones. It has been suggested that Tamm-Horsfall glycoprotein from stone–former’s urine has an abnormally low sialic acid (N-acetyleneuraminic acid) content and that this may make it more liable to polymerize and promote crystallization. Urinary sialic acid showed
marked inhibitory activity upon calcium oxalate crystal aggregation and growth at concentrations higher than 100 mg/dl.

Nephrocalcin is a urinary glycoprotein that occurs in calcium oxalate stone matrix and inhibits calcium oxalate crystal growth. This glycoprotein is made in the kidney and contains several residues of gamma-carboxy glutamic acid per molecule. Nakagawa and his colleagues found that the nephrocalcin isolated from the urine of calcium oxalate stone-formers lacks gamma-carboxyglutamic acid residues and forms interfacial films that are less stable than those formed by nephrocalcin from normal urine. Mutations in the gene directing the synthesis of this glycoprotein may well result in changes in amino acid composition, and thus contribute to the multifactorial aetiology of 'idiopathic' urinary stones.

Glycosaminoglycans and ribonucleic acids have also been proposed as physiological inhibitors of crystallization in urine. Glycosaminoglycans (GAG) are polysaccharide chains composed of repeating disaccharides of identical composition. Urinary GAG are degradation products of high molecular weight proteoglycans.

Inhibition of calcium oxalate crystallization by GAG is attributed to direct binding of calcium to GAG. The GAG chondroitin–A, chondroitin–C, heparan sulfate, dermatan sulfate, hyaluronic acid and keratan sulfate have all been shown to be inhibitors of calcium oxalate crystallization. Heparin, the only GAG which is not naturally present in urine, is the most potent inhibitor of
all GAG\textsuperscript{54}. The macromolecular inhibitors of crystallization bind to the surface of calcium oxalate crystals. The adsorbed layer of polyanions creates a negatively charged field around the crystals. This causes mutual repulsion between the coated crystals, which in turn, inhibits agglomeration and hence the formation of a particle which might become large enough to become trapped in the urinary passages and form the nucleus of a stone.

It has been proposed that normal urine contains surface active crystal poisons which protect against stone formation. Pyrophosphate ions, magnesium ions and citrate ions have attracted most interest in recent years. Magnesium\textsuperscript{55} and citrate\textsuperscript{56} appear to act partly by complexing stone constituents and partly by adsorption on to crystal surfaces where they act as crystal poisons, whereas pyrophosphate acts purely as a crystal poison\textsuperscript{44}.

3.1.5 Hypercalciuria

Disturbances of calcium metabolism are well documented in renal calcium stone disease and hypercalciuria is the most commonly occurring metabolic deviation\textsuperscript{57}. Hypercalciuria is defined as excretion of urinary calcium exceeding 200 mg in a 24 hour collection, and can be divided into three types: absorptive, resorptive and renal\textsuperscript{58}.

3.1.5.1 Absorptive hypercalciuria

Increased absorption of calcium from the gut results in increased circulating calcium, resulting in increased renal filtered load. The exact
mechanism is unknown but seems to be inherited in an autosomal dominant fashion, and the jejunal mucosa is hyper-responsive to vitamin D.

3.1.5.2 Resorptive hypercalciuria

Increased resorption of bone occurs as a result of primary hyperparathyroidism. This occurs in about 5% of patients with recurrent stone formation\textsuperscript{59}.

3.1.5.3 Renal hypercalciuria

Increased excretion of calcium in urine, results from impaired renal tubular absorption of calcium. This occurs in about 2% of patients with recurrent stone formation.

3.1.6 Hypocitraturia

Hypocitraturia\textsuperscript{57} is defined as urinary citrate excretion of < 250 mg in 24 hours. Higher urinary citrate prevents calcium stones by encouraging formation of soluble calcium citrate. It also reduces formation of urate stones by alkalinizing the urine\textsuperscript{60}. Hypocitraturia is present in about 40 percent of calcium stone formers, but in most cases the reason for this is unknown\textsuperscript{1}.

Urinary citrate is mainly derived endogenously through the tricarboxylic acid cycle and is excreted by renal tubular cells. Low urinary citrate excretion results from metabolic acidosis in conditions such as chronic diarrhoea, urinary diversion and distal renal tubular acidosis. The hypocitraturia of distal
renal tubular acidosis is due to increased reabsorption of citrate in the proximal tubule as a result of intracellular acidosis. Citrate excretion is also reduced because of acid retention in subjects on a high protein diet. Women excrete more citrate and have lower incidence of stone formation than men. Hormonal replacement therapy in postmenopausal women results in higher urinary calcium excretion, but it also increases urinary excretion of citrate and leads to net inhibition of crystal precipitation, thereby decreasing the risk of calcium stones. It has been reported that the urine of stone-formers showed low calcium oxalate solubility and normal crystal growth inhibition, but lacked the ability to inhibit crystal agglomeration and all the stone formers showed hypocitraturia.

3.1.7 Hyperuricosuria

The contribution of increased uric acid excretion to uric acid stone formation occurs mainly in patients on a high protein (purine) diet, which leads to the production of more acid urine and increases the risk of urate precipitation. Hyperuricosuria is less commonly due to a defect of urate metabolism. The principal determinant of uric acid crystallisation is the supersaturation of urine with respect to undissociated uric acid.

The urinary saturation of undissociated uric acid is dependent on pH and uric acid concentration. At urinary pH less than 5.5, the low solubility of uric acid leaves an excess of un-dissociated uric acid to cause uric acid lithiasis. At pH higher than 5.5, monosodium urate is particularly prone to
form uric acid crystals because of its low solubility and abundant sodium content of urine. Hyperuricosuria occurs in 10% of patients with calcium stones, where uric acid crystals form the nidus for deposition of calcium oxalate.

3.1.8 Hyperoxaluria

The excretion of oxalate is often mildly elevated in idiopathic stone formers. Most oxalate is derived from the metabolism of glycine and ascorbic acid. Man is to a certain extent protected against oxalate accumulation because oxalate absorption from the gastrointestinal tract is normally an inefficient process. Intestinal absorption of oxalate is normally low, but rises when dietary calcium content is reduced. It is also increased following small bowel resection and in Crohn's disease.

Recent research suggests that normal bowel colonization with the bacterium, *Oxalobacter formigenes* is an important determinant of urinary oxalate excretion, because this organism digests dietary oxalate, thereby reducing its absorption. Oral administration of *Oxalobacter* has been shown to decrease urinary oxalate concentration in animals and humans.

The two genetic types of primary hyperoxaluria are autosomal recessive and cause oxalate over production. Type-1 is the more severe form producing widespread tissue deposition of oxalate, early renal failure and nephrocalcinosis. It is due to a defect of the liver *transaminase* that converts glyoxylate to glycine, resulting in glyoxylate oxidation to oxalate and reduction.
to glycollate\textsuperscript{69–71}. The rarer type-2 is due to a deficiency of liver \textit{D-glycerate dehydrogenase} and is characterised by glyceraturia.\textsuperscript{72}

### 3.1.9 The influence of dietary factors

Nutrients influence stone formation both as promoters and inhibitors. Urinary stone formation is influenced by concentration of cereals and refined carbohydrates like lactose, sucrose and fructose\textsuperscript{73,74}. Carbohydrates may be oxalate precursors but the extent of their contribution is difficult to assess\textsuperscript{75}. A high intake of refined sugars may also increase the intestinal absorption of calcium\textsuperscript{1}.

Fat and purines are present in larger proportions in animal protein. Excessive intake of animal proteins aggravates the calcium oxalate stone formation due to increased urinary excretion of calcium, oxalate and uric acid\textsuperscript{75,76}. Sufficient calcium intake, i.e. 1200 mg per day, is crucial, because it allows for binding of oxalate at the intestinal level where by increases of urinary oxalate can be avoided\textsuperscript{77,78}. Too low an intake of calcium on the other hand, may increase the intestinal absorption and hence urinary excretion of oxalate. It is important, therefore, not to advise patients to cut out all dairy produce to correct their hyper calciuria, as they may end up with a higher risk of forming stones than when they started\textsuperscript{1}.

Dietary and urinary sodium is directly correlated with urinary calcium excretion. Lower urinary excretion of sodium reduces urinary calcium
excretion. High intake of salt leads to a renal leak of calcium and calcium excretion is increased by 30% by sodium chloride. High oxalate foods can enhance calcium oxalate stone formation. These include spinach, rhubarb, beetroot, nuts and chocolate. It is essential to include foods that have a high ratio of magnesium to calcium such as brown rice, bananas, oats, barley and soya and that are high in fiber such as oat bran, psyllium seed husk and flax seed meal. Low magnesium intake has been linked to stone formation. Magnesium supplementation may decrease the size of an existing stone and prevent further formations. It is reported that cranberry juice decreases oxalate and phosphate excretion and increases citrate excretion and fruits such as oranges and grapes are the main exogenous sources of urinary citrate.

3.1.10 Treatment of established urinary stone

Urinary stones can be either disrupted by extracorporeal shockwave lithotripsy or removed surgically. Minimally invasive nephroscopic removal has largely supplanted open surgical operations. Endoscopic removal is commonly combined with endoscopic stone disruption by either ultrasound, laser energy or electrohydraulically derived shock waves.

3.1.11 Medical treatment to prevent recurrent stones

Although these minimally invasive techniques are often the procedures of choice for the removal of stones, they do not prevent their
recurrence. Although extracorporeal shock wave lithotripsy has revolutionized the treatment of renal stones, many problems remain, such as the long-term renal damage, hypertension and symptomatic recurrences\textsuperscript{87,88}. Stone fragments that are retained after treatment may serve as nidi for the formation of new stones. A prophylactic approach that has not been extensively investigated is the use of chemical treatment to prevent or reduce the rate of stone recurrence in individuals subjected to lithotripsy.

Without biochemical screening and appropriate dietary and/or medical management, the patient will often return for further stone removal in the future, which can be uncomfortable for the patient and is expensive to perform. It is reported that the possibility of stone recurrence\textsuperscript{89} in a person is 67-100\% and that mere stone removal cannot provide complete cure for the disease. The ultimate control of urolithiasis requires a proper application of both the approaches of stone removal and drug therapy.

In recent years a variety of prophylactic agents have been used to reduce recurrences in patients suffering from calcium nephrolithiasis, including hydrochlorothiazide, orthophosphate, alkali–citrates and magnesium\textsuperscript{90}.

Drug therapy is mainly aimed at the inhibition of growth of the existing stone and the formation of new stone. From a naturopathic perspective, kidney stones that do not occur as a result of a genetic or metabolic disorder are considered to be a diet–related condition \textsuperscript{73-80}. Proper nutrition can
support healthy kidney function and may discourage stone formation\textsuperscript{81} and natural therapies may help ease the pain and spasm that accompanies stone passage.

**3.1.12 Traditional drugs for the treatment of urinary stones**

Barring D-Penicillamine and allopurinol for cystine and uric acid stones, no effective drug therapy is available in allopathy for the treatment or prevention of other types of stones. However, a large number of indigenous drugs have been used for this purpose in our country since ancient times\textsuperscript{91}. Sushruta (1000 B.C.) has very systemically described this disease and the medicines for its cure.

‘Pashanbheda’ is a drug mentioned in Ayurvedic system of medicine as a diuretic lithotriptic and is said to have property of breaking and disintegrating the stones. In recent years a number of proprietary drugs like ‘Cystone’ (Himalya Drug Co.) and ‘Calcury’ (Charak Pharmaceuticals) have been introduced for dissolving kidney calculi. *Saxifraga liqulata* and *Tribulus terrestris* (Gokhru) are two common herbs of these herbo–mineral formulations\textsuperscript{92}. Cystone is claimed to have properties of dissolving stones \textit{in situ} and expelling them without surgery. There are several reports in this regard but the only systematic clinical appraisal appears to be by Dandia et al.\textsuperscript{83} who concluded from their experimental study in rats and dogs that Cystone provide some protection against the growth of stones and their recurrence. It has repeatedly been shown that the majority of stone formers
excrete an abnormal urine and if its composition can be altered, the growth of the stones will cease. At the same time the chances of another stone formation could be minimised and dissolution of existing stones enhanced.

In addition to the above, a list of Indian medicinal plants, used for the treatment of urinary stones is presented in Table 3.0.

**Table 3.0**

<table>
<thead>
<tr>
<th>No</th>
<th>Biological name</th>
<th>Vernacular Name</th>
<th>Parts used</th>
<th>Uses</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td><em>Abutilon asiaticum</em></td>
<td>Tutti (Tam)</td>
<td>Leaves</td>
<td>For stones in bladder</td>
</tr>
<tr>
<td>2.</td>
<td><em>Artanema sesamoides</em></td>
<td>Kokilaksha (S)</td>
<td>Root</td>
<td>Stone</td>
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<td>3.</td>
<td><em>Asplenium falcatum</em></td>
<td>Nela panna maravara</td>
<td>Whole plant</td>
<td>Calculus</td>
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<td></td>
<td></td>
<td>(Mal)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4.</td>
<td><em>Barleria longiflora</em></td>
<td>Adyanda (S)</td>
<td>Root</td>
<td>Dropsy and Stone</td>
</tr>
<tr>
<td>5.</td>
<td><em>Caesal pinianuga</em></td>
<td>Kakamullu (Mal)</td>
<td>Root</td>
<td>Stone in bladder</td>
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<tr>
<td>7.</td>
<td><em>Chionachne koenigii</em></td>
<td>Gelagaddi (Tel)</td>
<td>Windeplant</td>
<td>Vesical Calculi</td>
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<td>8.</td>
<td><em>Citrus medica</em></td>
<td>Kadararanarathai (Tam)</td>
<td>Root</td>
<td>Urinary Calculus</td>
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<td>9.</td>
<td><em>Dendrophthoe elastica</em></td>
<td>Andagan (Tam)</td>
<td>Leaves</td>
<td>Stone in the bladder and kidney</td>
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<td></td>
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<td>Common Name</td>
<td>Part</td>
<td>Use</td>
</tr>
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<tr>
<td>10.</td>
<td><em>Dichrostachys cinerea</em></td>
<td>Vidattalai (Tam)</td>
<td>Root</td>
<td>Cure for stones and renal troubles</td>
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<td>11.</td>
<td><em>Didymocarpus pedicellata</em></td>
<td>Shilapushpa (S)</td>
<td>Leaves</td>
<td>Cure for stones in kidney and bladder</td>
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<td>12.</td>
<td><em>Homonoia riparia</em></td>
<td>Pashanabedaka (S)</td>
<td>Root</td>
<td>Stone in bladder</td>
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<td>13.</td>
<td><em>Lawsonia inermis</em></td>
<td>Marudondri (Tam)</td>
<td>Bark</td>
<td>Calculus affections</td>
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<td>14.</td>
<td><em>Mimosa pudica</em></td>
<td>Tottalvadi (Tam)</td>
<td>Root</td>
<td>Gravel complaints</td>
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<td>15.</td>
<td><em>Parmelia perlata</em></td>
<td>Kalpasi (Tam)</td>
<td>Whole plant</td>
<td>Calculi</td>
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<td><em>Prunus cerasoides</em></td>
<td>Padmaka (S)</td>
<td>Kernel</td>
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<tr>
<td>17.</td>
<td><em>Rotula aquatica</em></td>
<td>Pashanabheda (S)</td>
<td>Root</td>
<td>Stones in the bladder</td>
</tr>
<tr>
<td>18.</td>
<td><em>Saccharum spontaneum</em></td>
<td>Nanal (Tam)</td>
<td>Whole plant</td>
<td>Vesical calculi</td>
</tr>
<tr>
<td>19.</td>
<td><em>Spinacia oleracea</em></td>
<td>Vasaiylaikkirai (Tam)</td>
<td>Whole plant</td>
<td>Urinary calculi</td>
</tr>
<tr>
<td>20.</td>
<td><em>Tribulus alatus</em></td>
<td>Gokhuri – Kalan (H)</td>
<td>Fruits</td>
<td>Calculus affections</td>
</tr>
<tr>
<td>21.</td>
<td><em>Tribulus terrestris</em></td>
<td>Nerunji (Tam)</td>
<td>Fruits</td>
<td>Kidney diseases and gravel</td>
</tr>
</tbody>
</table>

S-Sanskrit; H-Hindi; Tam-Tamil; Mal-Malayam; Tel-Telugu.
3.2 PAST WORK ON INHIBITORY EFFECT OF SOME MEDICINAL PLANTS ON CALCIUM OXALATE CRYSTAL GROWTH AND AGGLOMERATION

Many remedies have been employed during ages to treat urinary stones. Most of the remedies were taken from plants and proved to be useful, though the rationale behind their use is not well established except for a few plants and some proprietary composite herbal drugs.

Only very limited work has been carried out on the growth of crystals which are the chemical constituents of urinary stones. Koide et al\(^4\) reported that Takusha (\textit{Alismatis rhizoma}) had strong inhibition using a seed crystal system \textit{in-vitro} and \textit{in-vivo}. Koji Suzuki et al\(^6\) evaluated the efficacy of “Takusha” in preventing the nucleation and growth \textit{in-vitro} of calcium oxalate crystals and have come to the conclusion that “Takusha” is a potent inhibitor of calcium oxalate crystal formation, aggregation and growth.

Ethanolic extract of \textit{Ammannia baccifera} was tested for its antiurolithiatic activity in male albino rats and was found to be effective in reducing the formation of stones and also in dissolving the pre-formed ones\(^5\). \textit{Phyllanthus niruri} is a plant used for years in Brazil to treat urinary calculi. Nishiura et al demonstrated that \textit{Phyllanthus niruri} had normalized elevated urinary calcium levels in calcium stone forming patients\(^6\). The \textit{in-vitro} effect of an aqueous extract of \textit{Phyllanthus niruri} on a model of calcium oxalate crystal endocytosis by Madin–Darby canine kidney cells has been
performed\textsuperscript{87} by Alexandre and his co–workers. The extract has exhibited a potent and effective non–concentration–dependent–inhibitory effect on the CaOX crystal internalization. The effect of an aqueous extract of \textit{P. niruri} on the urinary excretion of endogenous inhibitors of lithogenesis, citrate, magnesium and glycosaminoglycans was evaluated in a rat model of urolithiasis induced by the introduction of calcium oxalate seed into the bladder of adult male Wistar rats\textsuperscript{98}.

"Gahat" (\textit{Vigna unguiculata}) is a legume used for centuries in Nepal and Pakistan to treat the symptoms associated with urinary calculi. Schwartz \textit{et al} have evaluated the effect of Gahat consumption on 24–hour urine parameters in an attempt to assess its \textit{in–vivo} effect in normal volunteers\textsuperscript{99}. They have come to the conclusion that "Gahat" increased urinary magnesium and had no effect on other urine electrolytes.

The interaction of calcium oxalate crystals with renal epithelial cells is a critical event in kidney stone formation. The effect of aqueous extract from \textit{Herniaria hirsuta} on the adhesion of calcium oxalate monohydrate crystals to cultured renal cells has been performed by Atmani \textit{et al}\textsuperscript{100}. An extract of \textit{H. hirsuta} has promoted the nucleation of calcium oxalate crystals, increasing their number but decreasing their size\textsuperscript{101}. The prophylactic potential of a herbal decoction from \textit{H. hirsuta} has been evaluated by assessing the effect of oral administration in experimentally induced calcium oxalate nephrolithiasis in rats\textsuperscript{102}. Ethanol extract of the roots of \textit{Homonia riparia} was
tested for its antiurolithiatic activity against calcium oxalate and magnesium ammonium phosphate stones in male albino rats. The extract was found to be effective in reducing the formation and also in dissolving the pre-formed magnesium ammonium phosphate type of stones. The aqueous extract of the bark of Raphanus sativus was tested for its antiurolithiatic and diuretic activity. The urolithiasis was experimentally induced by implantation of zinc disc in the urinary bladder of rats. Significant decrease in the weight of stones was observed after treatment in animals which received aqueous extract in comparison with control groups.

3.3 THE NEED FOR IN-VITRO STUDIES

The formation of urinary crystals is an essential step in stone disease. Growth of urinary crystal in-vitro has become an integral part of the study of the nucleation and growth characteristics of crystals. Various methods and techniques are aimed at modelling crystallisation processes of urinary stone formation in-vitro. Crystallisation procedures carried out in aqueous solutions are likely to mimic crystalluria, corresponding to a free-particle mechanism. However, a specifically tailored flow technique of crystallisation in gels seems to be a reasonable model of stone formation, in accordance with the generally accepted fixed particle theory. Despite enormous research efforts to obtain a thorough knowledge on the formation and development of the uroliths, the mechanism of stone formation remains largely unexplained. The main reason for this is that the uroliths grow in-vivo.
and can not be observed directly and also *in-vivo* experiments are possible only to a limited extent. Thus the need to understand the situations that give rise to crystalline disease has necessitated the *in-vitro* investigations of the crystalline components present in the stones. Studies on the growth of urinary crystals in the presence of some substances which act as inhibitors or promoters of crystal growth may help to pick out potential substances for use by recurrent stone formers.  

### 3.3.1 Crystal growth techniques

It had been shown that the urinary stones grow in a gel like medium, which is probably one of the reasons for the radially striated growth of crystals found in urinary calculi. Thus gel seems to be an ideal medium to study the crystallization of urinary stone components as its viscous nature provides simulation of biological fluids in which it grows. Gel is defined as a colloidal system containing a liquid dispersed in a solid. It is recognized as a two-component system of semi solid nature, rich in liquid. Some of the preferred gel media are sodium metasilicate, agar–agar, gelatin, tetramethoxy silane, acrylamide and poly vinyl alcohol. Among these, the most commonly used gel is sodium metasilicate (SMS). When SMS goes into solution monosilicic acid is produced.

\[
\text{Na}_2\text{SiO}_3 + 3\text{H}_2\text{O} \rightarrow \text{H}_4\text{SiO}_4 + 2\text{NaOH}
\]
Monosilicic acid polymerizes with the liberation of water forming a three-dimensional network of Si–O links.

\[
\text{OH} \quad \text{OH} \\
\text{HO} \quad \text{Si} \quad \text{O} \quad \text{Si} \quad \text{O} \\
\text{O} \quad \text{O} \\
\text{HO} \quad \text{Si} \quad \text{O} \quad \text{Si} \quad \text{O} \\
\text{OH} \quad \text{OH}
\]

As the polymerization continues, water accumulates at the top of the gel surface. This phenomenon is known as syneresis\textsuperscript{111}.

Among the different types of gel methods, the chemical reaction and the solubility reduction techniques are widely used for the growth of biological crystals\textsuperscript{109}. In chemical reaction method, one of the reactants AX is incorporated inside the gel and the other reactant BY is allowed to diffuse from the top of the gel. Reaction takes place inside the gel and the insoluble product AB crystallizes out. The other byproduct XY must be water soluble

\[
AX + BY \rightarrow AB + XY
\]

In the solubility reduction method, the substance to be crystallized is incorporated into the gel and the solubility could be reduced by common ion effect or by withdrawing the solvent from the system, leading to supersaturation and the growth of the crystal.
3.4 SCOPE OF THE PRESENT STUDY

The problem of urinary calculus is a very ancient one and many remedies have been employed during the ages. These stones are found in all parts of the urinary tract - the kidney, the ureter and the bladder and may vary considerably in size, shape, hardness and chemical composition. The most important constituents of stones include calcium oxalate, calcium phosphate, uric acid, magnesium ammonium phosphate, sodium and ammonium urates, cystine, xanthine etc. However, the most common type is calcium oxalate stones. Besides chemical and micro chemical analysis, X-ray, IR and SEM analysis have been found to be highly efficient in studying and understanding the composition of human stones. From time immemorial indigenous drugs have provided remedy for a large variety of human ailments and urinary stones are no exception.

A number of Ayurvedic drugs have been used in India and elsewhere and claims of efficient cures have been made in the treatment of urinary stones. Yet there is no scientific proof based on systematic study using these plant drugs, their composition or pharmacological effects. In this circumstances an attempt has been made to evaluate the inhibitory effect of methanolic and aqueous extracts of five medicinal plants viz. *Dichrostachys cinerea*, *Hemidesmus indicus*, *Parmelia perlata*, *Sida acuta* and *Sida cordata* on calcium oxalate crystal growth *in-vitro*. This *in-vitro* crystal growth experiments are expected to provide experimental evidence for the curative claims of the drugs against urinary stones.
3.5 RESULTS

3.5.1 Lengths of crystal columns

The commonly seen calcium oxalate crystals in human urine namely whewellite (calcium oxalate monohydrate) were grown *in-vitro* in silica gel medium in Hane's tubes by single diffusion method\textsuperscript{105}. The calcium oxalate crystals appeared as cloudy precipitate and in due course, the depth of the column was found to increase. The thickness (length) of crystal column was measured in centimetre on days 1, 3, 7, 10, 15, 20, 25 and 30. The inhibitory effect of methanolic and aqueous extracts of medicinally important parts of *Dichrostachys cinerea, Hemidesmus indicus, Parmelia perlata, Sida acuta* and *Sida cordata* were studied at 20 mg/5 ml and 10 mg/5 ml concentrations.

The inhibitory effect of the methanolic extract of the root of *D. cinerea* on calcium oxalate crystal growth has been studied *in-vitro* at 20 mg/5 ml and 10 mg/5 ml doses and the results are presented in Table 3.1. and Fig. 3.1. Concentration independent inhibitory effect was observed for *D. cinerea* with regard to the lengths of crystal columns, where the lengths of crystal columns were almost equal for higher and lower concentrations, but less than the size of crystal columns in the control set up. Reductions in the lengths of crystal columns were observed even on the first day of the experiment for the methanolic extract of *D. cinerea* and the reduction in growth rate was more significant from fifth day onwards. The mean length of crystal column in the
Table 3.1

Effect of methanolic extract of *Dichrostachys cinerea* root on the lengths of calcium oxalate crystal columns.

<table>
<thead>
<tr>
<th>Days</th>
<th>Control</th>
<th><em>D. cinerea</em> 20 mg/5 ml</th>
<th><em>D. cinerea</em> 10 mg/5 ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1.783 ± 0.08</td>
<td>1.683 ± 0.02</td>
<td>1.700 ± 0.00</td>
</tr>
<tr>
<td>3</td>
<td>2.200 ± 0.05</td>
<td>2.100 ± 0.00</td>
<td>2.116 ± 0.02</td>
</tr>
<tr>
<td>7</td>
<td>2.716 ± 0.01</td>
<td>2.416 ± 0.02 ***</td>
<td>2.400 ± 0.03 ***</td>
</tr>
<tr>
<td>10</td>
<td>3.016 ± 0.03</td>
<td>2.566 ± 0.02 ***</td>
<td>2.583 ± 0.05 ***</td>
</tr>
<tr>
<td>15</td>
<td>3.266 ± 0.03</td>
<td>2.733 ± 0.03 ***</td>
<td>2.700 ± 0.05 ***</td>
</tr>
<tr>
<td>20</td>
<td>3.483 ± 0.04</td>
<td>2.900 ± 0.03 ***</td>
<td>2.900 ± 0.05 ***</td>
</tr>
<tr>
<td>25</td>
<td>3.533 ± 0.04</td>
<td>2.900 ± 0.03 ***</td>
<td>2.900 ± 0.05 ***</td>
</tr>
<tr>
<td>30</td>
<td>3.483 ± 0.04</td>
<td>2.900 ± 0.03 ***</td>
<td>2.900 ± 0.05 ***</td>
</tr>
</tbody>
</table>

Lengths of crystal columns are given in cm. All values are mean ± SEM of six experiments in each set * P < 0.05; **P < 0.01; *** P < .001.
Fig. 3.1. Effect of methanolic extract of *D. cinerea* root on the lengths of calcium oxalate crystal columns.
control set up was 3.48 cm on the 30th day when ethanol was used as the control. The mean lengths of crystal columns in the extract set up were 2.9 cm for both the concentrations on the 30th day of the experiment.

Table 3.2 and Fig. 3.2 show the effect of aqueous extract of \textit{D. cinerea} on calcium oxalate crystal growth \textit{in-vitro} at two different concentrations \textit{viz.}, 20 mg/5 ml and 10 mg/5 ml. It is evident that marked reductions in crystal growth could be seen only from 20th day onwards for both 20 mg/5 ml and 10 mg/5 ml concentrations. The reduction was also concentration independent as, in the case with methanolic extract of \textit{D. cinerea}.

Concentration dependent inhibitory effect was observed in both methanolic and aqueous extracts of \textit{H. indicus}. In the case of methanolic extracts both 20 mg/5 ml and 10 mg/5 ml concentrations did not produce any significant change on crystal growth up to the 3rd day. On the third day the growth was almost equal in control and drug added tubes (Table 3.3 and Fig. 3.3) From the 5th day onwards significant decrease in growth rate was observed in tubes containing methanolic extracts. The mean length of crystal column in the control set up was 3.48 cm on the 30th day of the experiment when ethanol was used as the control. The mean lengths of crystal columns in the extract setup were 3.00 cm (20 mg/5 ml) and 3.13 cm (10 mg/5 ml) for methanolic extracts. The degree of inhibition was more with 20 mg/5 ml concentration.
Table 3.2

Effect of aqueous extract of *Dichrostachys cinerea* root on the lengths of calcium oxalate crystal columns.

<table>
<thead>
<tr>
<th>Days</th>
<th>Control</th>
<th><em>D. cinerea</em></th>
<th><em>D. cinerea</em></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>20 mg/5 ml</td>
<td>10 mg/5 ml</td>
</tr>
<tr>
<td>1.</td>
<td>1.733 ± 0.02</td>
<td>1.783 ± 0.04</td>
<td>1.716 ± 0.07</td>
</tr>
<tr>
<td>3.</td>
<td>2.566 ± 0.02</td>
<td>2.583 ± 0.03</td>
<td>2.566 ± 0.05</td>
</tr>
<tr>
<td>7.</td>
<td>3.133 ± 0.02</td>
<td>3.150 ± 0.03</td>
<td>3.133 ± 0.03</td>
</tr>
<tr>
<td>10.</td>
<td>3.616 ± 0.02</td>
<td>3.650 ± 0.03</td>
<td>3.633 ± 0.03</td>
</tr>
<tr>
<td>15.</td>
<td>4.016 ± 0.02</td>
<td>4.050 ± 0.04</td>
<td>4.033 ± 0.04</td>
</tr>
<tr>
<td>20.</td>
<td>4.683 ± 0.04</td>
<td>4.400 ± 0.02**</td>
<td>4.466 ± 0.04*</td>
</tr>
<tr>
<td>25.</td>
<td>3.966 ± 0.07</td>
<td>3.666 ± 0.02**</td>
<td>3.633 ± 0.03**</td>
</tr>
<tr>
<td>30.</td>
<td>3.966 ± 0.07</td>
<td>3.650 ± 0.02**</td>
<td>3.650 ± 0.03**</td>
</tr>
</tbody>
</table>

Lengths of crystal columns are given in cm. All values are mean ± SEM of six experiments in each set * P < 0.05; ** P < 0.01; *** P < .001.
Fig. 3.2. Effect of aqueous extract of *D. cinerea* root on the lengths of calcium oxalate crystal columns.
Table 3.3

Effect of methanolic extract of *Hemidesmus indicus* root on the lengths of calcium oxalate crystal columns.

<table>
<thead>
<tr>
<th>Days</th>
<th>Control</th>
<th><em>H. indicus</em> 20 mg/5 ml</th>
<th><em>H. indicus</em> 10 mg/5 ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>1.783 ± 0.08</td>
<td>1.783 ± 0.01</td>
<td>1.800 ± 0.00</td>
</tr>
<tr>
<td>3.</td>
<td>2.200 ± 0.05</td>
<td>2.166 ± 0.03</td>
<td>2.233 ± 0.01</td>
</tr>
<tr>
<td>7.</td>
<td>2.716 ± 0.01</td>
<td>2.450 ± 0.02***</td>
<td>2.583 ± 0.08</td>
</tr>
<tr>
<td>10.</td>
<td>3.016 ± 0.03</td>
<td>2.616 ± 0.01***</td>
<td>2.766 ± 0.08*</td>
</tr>
<tr>
<td>15.</td>
<td>3.266 ± 0.03</td>
<td>2.866 ± 0.01***</td>
<td>3.016 ± 0.08*</td>
</tr>
<tr>
<td>20.</td>
<td>3.483 ± 0.04</td>
<td>3.000 ± 0.00***</td>
<td>3.116 ± 0.08**</td>
</tr>
<tr>
<td>25.</td>
<td>3.533 ± 0.04</td>
<td>3.000 ± 0.00***</td>
<td>3.133 ± 0.08**</td>
</tr>
<tr>
<td>30.</td>
<td>3.483 ± 0.04</td>
<td>3.000 ± 0.00***</td>
<td>3.133 ± 0.08*</td>
</tr>
</tbody>
</table>

Lengths of the crystal columns are given in centimeter. All values are mean ± SEM of six experiments in each set. * P < 0.05; ** P < 0.01; *** P < 0.001.
Fig. 3.3. Effect of methanolic extract of *H. indicus* root on the lengths of calcium oxalate crystal columns.
Aqueous extracts of *H. indicus* did not produce significant reductions in lengths of crystal columns in any of the days (Table 3.4 and Fig. 3.4). The mean length of crystal column on the 30th day in the control setup was 3.97 cm when distilled water was used as the control. The mean lengths of crystal columns on the same day in the extracts set up were 3.87 cm (20 mg/5 ml) and 3.90 cm (10 mg/5 ml) for aqueous extracts. Here also the degree of inhibition was more with 20 mg/5 ml concentration.

The inhibitory effect of methanolic and aqueous extracts of the plant body of *P. perlata* have been studied at 20 mg/5 ml and 10 mg/5 ml doses and the results are presented in Fig. 3.5 and 3.6 and Tables 3.5 and 3.6. The reductions in growth rate were not significant in both the extracts. In the case of methanolic extracts the lengths of crystal columns were almost equal in control and drug added tubes up to the 7th day of the experiment. On the 10th day a slight decrease in growth rate was observed in drug added tubes compared to that of the control. The growth rate slowed down further on the succeeding days. However, marked reduction in crystal growth was observed only in tubes containing 20 mg/5 ml of the drug and in tubes containing 10 mg/5 ml of *P. perlata* there was no appreciable difference in lengths of crystal columns from the control (Table 3.5 and Fig. 3.5).

In the case of aqueous extract of *P. perlata* very minute reductions in growth rate were observed from 20th day onwards in tubes containing 20 mg/5 ml of the extract (Table 3.6 and Fig. 3.6).
Table 3.4

Effect of aqueous extracts of *Hemidesmus indicus* root
on the lengths of calcium oxalate crystal columns

<table>
<thead>
<tr>
<th>Days</th>
<th>Control</th>
<th><em>H. indicus</em> 20 mg/5 ml</th>
<th><em>H. indicus</em> 10 mg/5 ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1.733 ± 0.02</td>
<td>1.666 ± 0.02</td>
<td>1.700 ± 0.06</td>
</tr>
<tr>
<td>3</td>
<td>2.566 ± 0.02</td>
<td>2.550 ± 0.03</td>
<td>2.566 ± 0.04</td>
</tr>
<tr>
<td>7</td>
<td>3.133 ± 0.02</td>
<td>3.150 ± 0.03</td>
<td>3.116 ± 0.04</td>
</tr>
<tr>
<td>10</td>
<td>3.616 ± 0.02</td>
<td>3.583 ± 0.04</td>
<td>3.616 ± 0.04</td>
</tr>
<tr>
<td>15</td>
<td>4.016 ± 0.02</td>
<td>4.000 ± 0.03</td>
<td>4.016 ± 0.04</td>
</tr>
<tr>
<td>20</td>
<td>4.683 ± 0.04</td>
<td>4.516 ± 0.02*</td>
<td>4.600 ± 0.06</td>
</tr>
<tr>
<td>25</td>
<td>3.966 ± 0.07</td>
<td>3.866 ± 0.03</td>
<td>3.916 ± 0.04</td>
</tr>
<tr>
<td>30</td>
<td>3.966 ± 0.07</td>
<td>3.866 ± 0.03</td>
<td>3.900 ± 0.05</td>
</tr>
</tbody>
</table>

Lengths of the crystal columns are given in centimeter. All values are mean ± SEM of six experiments in each set. * P < 0.05; ** P < 0.01; *** P < 0.001.
Fig. 3.4. Effect of aqueous extract of *H. indicus* root on the lengths of calcium oxalate crystal columns.
Table 3.5

Effect of methanolic extract of *Parmelia perlata*

on the lengths of calcium oxalate crystal columns.

<table>
<thead>
<tr>
<th>Days</th>
<th>Control</th>
<th><em>P. perlata</em> 20 mg/5 ml</th>
<th><em>P. perlata</em> 10 mg/5 ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1.783 ± 0.08</td>
<td>1.783 ±0.05</td>
<td>1.716 ±0.04</td>
</tr>
<tr>
<td>3</td>
<td>2.200 ± 0.05</td>
<td>2.166 ±0.05</td>
<td>2.166 ±0.04</td>
</tr>
<tr>
<td>7</td>
<td>2.716 ± 0.01</td>
<td>2.733 ± 0.03</td>
<td>2.716 ±0.04</td>
</tr>
<tr>
<td>10</td>
<td>3.016 ± 0.03</td>
<td>2.950 ±0.02</td>
<td>2.983 ±0.02</td>
</tr>
<tr>
<td>15</td>
<td>3.266 ± 0.03</td>
<td>3.150 ±0.03*</td>
<td>3.233 ±0.02</td>
</tr>
<tr>
<td>20</td>
<td>3.483 ± 0.04</td>
<td>3.200 ±0.02**</td>
<td>3.350 ±0.01*</td>
</tr>
<tr>
<td>25</td>
<td>3.533 ± 0.04</td>
<td>3.216 ±0.02***</td>
<td>3.366 ±0.00**</td>
</tr>
<tr>
<td>30</td>
<td>3.483 ± 0.04</td>
<td>3.133 ± 0.02***</td>
<td>3.316 ±0.00**</td>
</tr>
</tbody>
</table>

Length of the crystal columns are given in centimeter. All values are mean ± SEM of six experiment in each set * P < 0.05; ** P<0.01; ***P < 0.001.
Fig. 3.5. Effect of methanolic extract of *P. perlata* on the lengths of calcium oxalate crystal columns.
Table 3.6

Effect of aqueous extract of *Parmelia perlata*

on the lengths of calcium oxalate crystal columns

<table>
<thead>
<tr>
<th>Days</th>
<th>Control</th>
<th><em>P. perlata</em> 20 mg/5 ml</th>
<th><em>P. perlata</em> 10 mg/5 ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1.733 ± 0.02</td>
<td>1.720 ± 0.03</td>
<td>1.730 ± 0.04</td>
</tr>
<tr>
<td>3</td>
<td>2.566 ± 0.02</td>
<td>2.556 ± 0.03</td>
<td>2.580 ± 0.04</td>
</tr>
<tr>
<td>7</td>
<td>3.133 ± 0.02</td>
<td>3.120 ± 0.04</td>
<td>3.150 ± 0.03</td>
</tr>
<tr>
<td>10</td>
<td>3.616 ± 0.02</td>
<td>3.622 ± 0.02</td>
<td>3.650 ± 0.04</td>
</tr>
<tr>
<td>15</td>
<td>4.016 ± 0.02</td>
<td>4.024 ± 0.02</td>
<td>4.050 ± 0.03</td>
</tr>
<tr>
<td>20</td>
<td>4.683 ± 0.04</td>
<td>4.500 ± 0.02</td>
<td>4.530 ± 0.03</td>
</tr>
<tr>
<td>25</td>
<td>3.966 ± 0.07</td>
<td>3.830 ± 0.03</td>
<td>3.930 ± 0.02</td>
</tr>
<tr>
<td>30</td>
<td>3.966 ± 0.07</td>
<td>3.833 ± 0.03</td>
<td>3.930 ± 0.02</td>
</tr>
</tbody>
</table>

Lengths of the crystal columns are given in centimeter. All values are mean ± SEM of six experiment in each set * P < 0.05; ** P<0.01; ***P < 0.001.
Fig. 3.6. Effect of aqueous extract of *P. perlata* on the lengths of calcium oxalate crystal columns.
The effect of methanolic extract of *Sida acuta* on the length of crystal column is presented in Table 3.7 and Fig. 3.7. Concentration dependent inhibitory effect was observed in the case of methanolic extract of *S. acuta* where the degree of inhibition was more with 20 mg/5 ml concentration. Reductions in the lengths of crystal columns were observed even on the first day of the experiment for methanolic extract of *S. acuta* and the reduction in growth rate was more significant from fifth day onwards. The mean length of crystal columns in the control set up was 3.48 cm on the 30\textsuperscript{th} day of the experiment when ethanol was used as the control. The mean lengths of crystal columns in the extract set up were 2.88 cm (20 mg/5 ml) and 2.93 cm (10 mg/5 ml) for methanolic extracts.

In the case of aqueous extract of *S. acuta*, the lengths of crystal columns were almost equal for higher and lower concentrations but slightly less than the length of crystal column in the control set up. Aqueous extracts of *S. acuta* did not produce significant reductions in the lengths of crystal columns. The mean length of crystal columns in the control set up was 3.97 cm on the 30\textsuperscript{th} day when distilled water was used as the control. The mean lengths of crystal columns in the extract added tubes were 3.83 cm, (20 mg/5 ml) and 3.83 cm (10 mg/5 ml) for aqueous extracts (Table 3.8 and Fig. 3.8).

Inhibitory effect of methanolic extract of *S. cordata* was studied at two different concentrations and the results are presented in Table 3.9. and Fig. 3.9. Graphical representation of the results (Fig. 3.9). shows clearly that,
Table 3.7

Effect of methanolic extract of *Sida acuta* root
on the lengths of calcium oxalate crystal columns.

<table>
<thead>
<tr>
<th>Days</th>
<th>Control</th>
<th><em>Sida acuta</em> 20 mg/5 ml</th>
<th><em>Sida acuta</em> 10 mg/5 ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>1.783 ± 0.08</td>
<td>1.700 ± 0.00</td>
<td>1.633 ± 0.03</td>
</tr>
<tr>
<td>3.</td>
<td>2.200 ± 0.05</td>
<td>2.150 ± 0.00</td>
<td>2.133 ± 0.03</td>
</tr>
<tr>
<td>7.</td>
<td>2.716 ± 0.01</td>
<td>2.383 ± 0.01***</td>
<td>2.383 ± 0.03***</td>
</tr>
<tr>
<td>10.</td>
<td>3.016 ± 0.03</td>
<td>2.533 ± 0.03***</td>
<td>2.550 ± 0.05***</td>
</tr>
<tr>
<td>15.</td>
<td>3.266 ± 0.03</td>
<td>2.783 ± 0.01***</td>
<td>2.783 ± 0.08**</td>
</tr>
<tr>
<td>20.</td>
<td>3.483 ± 0.04</td>
<td>2.883 ± 0.01***</td>
<td>2.933 ± 0.08**</td>
</tr>
<tr>
<td>25.</td>
<td>3.533 ± 0.04</td>
<td>2.883 ± 0.01***</td>
<td>2.933 ± 0.08**</td>
</tr>
<tr>
<td>30.</td>
<td>3.483 ± 0.04</td>
<td>2.883 ± 0.01***</td>
<td>2.933 ± 0.08**</td>
</tr>
</tbody>
</table>

Lengths of crystal columns are given in centimeter. All values are mean ± SEM of six experiments in each set * P< 0.05; **P<0.01; ***P<0.001.
Fig. 3.7. Effect of methanolic extract of *S. acuta* root on the lengths of calcium oxalate crystal columns.
Table 3.8

Effect of aqueous extract of *Sida acuta* root
on the lengths of calcium oxalate crystal columns.

<table>
<thead>
<tr>
<th>Days</th>
<th>Control</th>
<th><em>S. acuta</em> 20 mg/5 ml</th>
<th><em>S. acuta</em> 10 mg/5 ml</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.</td>
<td>1.733 ± 0.02</td>
<td>1.700 ± 0.02</td>
<td>1.750 ± 0.03</td>
</tr>
<tr>
<td>3.</td>
<td>2.566 ± 0.02</td>
<td>2.533 ± 0.03</td>
<td>2.583 ± 0.03</td>
</tr>
<tr>
<td>7.</td>
<td>3.133 ± 0.02</td>
<td>3.083 ± 0.04</td>
<td>3.166 ± 0.03</td>
</tr>
<tr>
<td>10.</td>
<td>3.616 ± 0.02</td>
<td>3.566 ± 0.03</td>
<td>3.650 ± 0.03</td>
</tr>
<tr>
<td>15.</td>
<td>4.016 ± 0.02</td>
<td>3.950 ± 0.03</td>
<td>4.050 ± 0.04</td>
</tr>
<tr>
<td>20.</td>
<td>4.683 ± 0.04</td>
<td>4.566 ± 0.02</td>
<td>4.566 ± 0.04</td>
</tr>
<tr>
<td>25.</td>
<td>3.966 ± 0.07</td>
<td>3.833 ± 0.02</td>
<td>3.766 ± 0.03</td>
</tr>
<tr>
<td>30.</td>
<td>3.966 ± 0.07</td>
<td>3.833 ± 0.03</td>
<td>3.833 ± 0.04</td>
</tr>
</tbody>
</table>

Lengths of crystal columns are given in centimeter. All values are mean ± SEM of six experiments in each set * P<0.05; **P<0.01; ***P<0.001.
Fig. 3.8. Effect of aqueous extract of *S. acuta* root on the lengths of calcium oxalate crystal columns.
Table 3.9

Effect of methanolic extract of aerial parts of *Sida cordata* on the lengths of calcium oxalate crystal columns

<table>
<thead>
<tr>
<th>Days</th>
<th>Control</th>
<th><em>S. cordata</em> 20 mg/5 ml</th>
<th><em>S. cordata</em> 10 mg/5 ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>1.783 ± 0.08</td>
<td>1.716 ±0.08</td>
<td>1.766 ±0.05</td>
</tr>
<tr>
<td>3.</td>
<td>2.200 ± 0.05</td>
<td>2.116 ±0.05</td>
<td>2.200 ±0.05</td>
</tr>
<tr>
<td>7.</td>
<td>2.716 ± 0.01</td>
<td>2.400 ±0.02***</td>
<td>2.450± 0.02***</td>
</tr>
<tr>
<td>10.</td>
<td>3.016 ± 0.03</td>
<td>2.600 ±0.02***</td>
<td>2.600±0.02***</td>
</tr>
<tr>
<td>15.</td>
<td>3.266 ± 0.03</td>
<td>2.850 ±0.02***</td>
<td>2.866 ±0.02***</td>
</tr>
<tr>
<td>20.</td>
<td>3.483 ± 0.04</td>
<td>3.000 ±0.03***</td>
<td>3.016±0.02***</td>
</tr>
<tr>
<td>25.</td>
<td>3.533 ± 0.04</td>
<td>3.000 ±0.03***</td>
<td>3.016±0.03***</td>
</tr>
<tr>
<td>30.</td>
<td>3.483 ± 0.04</td>
<td>3.000 ±0.03***</td>
<td>3.016±0.03***</td>
</tr>
</tbody>
</table>

Lengths of crystal columns are given in centimeter. All values are mean ± SEM of six experiments in each set * P< 0.05; **p.<0.01; ***P<0.001
3.9. Effect of methanolic extract of aerial parts of *S. cordata* on the lengths of calcium oxalate crystal columns.
there is not much difference between higher and lower concentration with regard to the lengths of crystal columns, but both 20 mg and 10 mg levels showed statistically significant reductions in growth rate compared to that of the control. The mean length of crystal columns in the control set up was 3.48 cm when ethanol was used as the control and the mean lengths of crystal columns in the drug added tubes were 3.00 cm (20 mg/5 ml) and 3.01 cm (10 mg/5 ml) for S. cordata on the 30th day.

The graphical representation of aqueous extract of S. cordata (Fig. 3.10 and Table 3.10) also show that the lengths of crystal columns in 20 mg level (3.81 cm) and 10 mg level (3.90 cm) do not differ much from the control (3.97 cm) on the 30th day or the preceeding days.

3.5.2 Size of individual crystals

Calcium oxalate monohydrate crystals were grown in–vitro in silica gel medium in Hane’s tubes by single diffusion method and the sizes of the crystals were monitored on days 1, 3, 7, 14, 21 and 28. For measuring the sizes of the crystals, the crystals were pipetted out using a micropipette and placed on a glass slide. The adhering gel was homogenized gently with distilled water, and a cover slip was placed on top and viewed under a high power lens. The size of the crystal was measured using a micrometer and the effects of methanolic and aqueous extracts of medicinally important parts of Dichrostachys cinerea, Hemidesmus indicus, Parmelia perlata, Sida acuta and Sida cordata on the size of the crystals were studied at
Table 3.10

Effect of aqueous extract of aerial parts of *Sida cordata* on the lengths of calcium oxalate crystal columns

<table>
<thead>
<tr>
<th>Days</th>
<th>Control</th>
<th><em>S. cordata</em> 20 mg/5 ml</th>
<th><em>S. cordata</em> 10 mg/5 ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>1.733 ± 0.02</td>
<td>1.700 ± 0.02</td>
<td>1.700 ± 0.04</td>
</tr>
<tr>
<td>3.</td>
<td>2.566 ± 0.02</td>
<td>2.533 ± 0.02</td>
<td>2.566 ± 0.03</td>
</tr>
<tr>
<td>7.</td>
<td>3.133 ± 0.02</td>
<td>3.100 ± 0.03</td>
<td>3.133 ± 0.04</td>
</tr>
<tr>
<td>10.</td>
<td>3.616 ± 0.02</td>
<td>3.616 ± 0.03</td>
<td>3.633 ± 0.04</td>
</tr>
<tr>
<td>15.</td>
<td>4.016 ± 0.02</td>
<td>4.016 ± 0.03</td>
<td>4.016 ± 0.03</td>
</tr>
<tr>
<td>20.</td>
<td>4.683 ± 0.04</td>
<td>4.466 ± 0.02</td>
<td>4.483 ± 0.03</td>
</tr>
<tr>
<td>25.</td>
<td>3.966 ± 0.07</td>
<td>3.900 ± 0.02</td>
<td>3.900 ± 0.02</td>
</tr>
<tr>
<td>30.</td>
<td>3.966 ± 0.07</td>
<td>3.816 ± 0.02</td>
<td>3.900 ± 0.02</td>
</tr>
</tbody>
</table>

Lengths of crystal columns are given in centimeter. All values are mean ± SEM of six experiments in each set * P< 0.05; ** P< 0.01; *** P< 0.001.
Fig. 3.10. Effect of aqueous extract of aerial parts of *S. cordata* on the lengths of calcium oxalate crystal columns.
20 mg/5 ml and 10 mg/5 ml concentration and are presented in Tables 3.11–3.20. and Fig. 3.11.–3.20. At the end of the 30th day, the crystals were cleared off the gel by repeated washing with distilled water and filtered through filter paper. The crystals thus obtained were air-dried and kept in clean dry bottles.

The effect of methanolic extract of *D. cinerea* on size of individual calcium oxalate crystal was monitored and the results are presented in Table 3.11 and Fig. 3.11. The drug was added at 20 mg/5 ml and 10 mg/5 ml concentrations. The drug at 20 mg and 10 mg levels enhanced the crystal growth for first few days and thereafter retardation in crystal growth was observed. However marked reduction in crystal growth was produced by both the concentrations of the drug from 7th day onwards. The reduction in growth was concentration dependent and 20 mg/5 ml was more effective than 10 mg/5 ml. The reduction from 14th to 28th day was highly significant for 20 mg/5 ml dose.

The effect of aqueous extract of *D. cinerea* on in-vitro crystal growth was studied at 20 mg/5 ml and 10 mg/5 ml concentration and the results are presented in Table 3.12 and Fig. 3.12. The drug at both the concentrations produced inhibition of crystal growth to a statistically significant level from 1st to 28th day. Graphical representation of the results (Fig. 3.12) clearly shows that maximum inhibition was for 20 mg/5 ml concentration. The mean size of grown crystals on 28th day in the control set up was 294 μm when distilled water was used as the control. The mean size of the crystals in the
Table 3.11

Effect of methanolic extract of *Dichrostachys cinerea* root on the size of calcium oxalate crystals.

<table>
<thead>
<tr>
<th>Days</th>
<th>Control</th>
<th><em>D. cinerea</em> 20 mg/5 ml</th>
<th><em>D. cinerea</em> 10 mg/5 ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>48.67 ± 5.06</td>
<td>80.00 ± 3.72**</td>
<td>84.12 ± 4.62**</td>
</tr>
<tr>
<td>3.</td>
<td>120.00 ± 6.87</td>
<td>156.00 ± 5.06**</td>
<td>150.00 ± 6.65*</td>
</tr>
<tr>
<td>7.</td>
<td>168.00 ± 4.45</td>
<td>130.00 ± 2.50***</td>
<td>132.00 ± 2.59***</td>
</tr>
<tr>
<td>21.</td>
<td>174.00 ± 6.58</td>
<td>126.00 ± 3.41**</td>
<td>160.00 ± 3.98</td>
</tr>
<tr>
<td>28.</td>
<td>180.00 ± 7.57</td>
<td>126.80 ± 2.50**</td>
<td>148.00 ± 3.44*</td>
</tr>
</tbody>
</table>

Sizes of crystals are given in microns. All values are mean ± SEM of six experiments in each set * P< 0.05 ; **P<0.01; ***P<0.001.
Fig. 3.11. Effect of methanolic extract of *D. cinerea* root on the size of the calcium oxalate crystals.
Table 3.12

Effect of aqueous extract of *Dichrostachys cinerea* root on the size of calcium oxalate crystals.

<table>
<thead>
<tr>
<th>Days</th>
<th>Control</th>
<th><em>D. cinerea</em> 20 mg/5 ml</th>
<th><em>D. cinerea</em> 10 mg/5 ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>90.37 ± 6.70</td>
<td>60.17 ± 5.03*</td>
<td>66.00 ± 3.74*</td>
</tr>
<tr>
<td>3.</td>
<td>204.44 ± 5.12</td>
<td>150.50 ± 2.66***</td>
<td>174.33 ± 3.03**</td>
</tr>
<tr>
<td>7.</td>
<td>312.00 ± 4.43</td>
<td>170.00 ± 2.49***</td>
<td>216.83 ± 3.49***</td>
</tr>
<tr>
<td>14.</td>
<td>222.00 ± 8.34</td>
<td>162.00 ± 5.08**</td>
<td>188.49 ± 2.62*</td>
</tr>
<tr>
<td>21.</td>
<td>228.00 ± 6.86</td>
<td>164.50 ± 3.41***</td>
<td>188.73 ± 1.42**</td>
</tr>
<tr>
<td>28.</td>
<td>294.00 ± 8.54</td>
<td>172.44±4.21***</td>
<td>224.0±02.13***</td>
</tr>
</tbody>
</table>

Sizes of crystals are given in microns. All values are mean ± SEM of six experiments in each set * P< 0.05 ; **P<0.01; ***P<0.001
Fig. 3.12. Effect of aqueous extract of *D. cinerea* root on the size of the calcium oxalate crystals.
Table 3.13

Effect of methanolic extract of *Hemidesmus indicus* root
on the size of calcium oxalate crystals.

<table>
<thead>
<tr>
<th>Days</th>
<th>Control</th>
<th><em>H. indicus</em> 20 mg/5 ml</th>
<th><em>H. indicus</em> 10 mg/5 ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>48.67 ± 5.06</td>
<td>90.00 ± 2.95***</td>
<td>78.00 ± 2.49**</td>
</tr>
<tr>
<td>3</td>
<td>120.00 ± 6.87</td>
<td>126.14 ± 3.48</td>
<td>132.36 ± 3.03</td>
</tr>
<tr>
<td>7</td>
<td>168.00 ± 4.45</td>
<td>168.26 ± 2.90</td>
<td>180.23 ± 2.91</td>
</tr>
<tr>
<td>14</td>
<td>210.85 ± 6.58</td>
<td>144.00 ± 3.84***</td>
<td>150.14 ± 3.43***</td>
</tr>
<tr>
<td>21</td>
<td>174.00 ± 6.58</td>
<td>132.12 ± 2.90**</td>
<td>132.00 ± 2.63**</td>
</tr>
<tr>
<td>28</td>
<td>180.00 ± 7.57</td>
<td>150.00 ± 2.46*</td>
<td>162.00 ± 2.95</td>
</tr>
</tbody>
</table>

Sizes of crystals are given in microns. All values are mean ± SEM of six experiments in each set * P< 0.05; ** P< 0.01; *** P< 0.001.
Fig. 3.13. Effect of methanolic extract of *H. indicus* root on the size of the calcium oxalate crystals.
Table 3.14

Effect of aqueous extract of *Hemidesmus indicus* root on the size of calcium oxalate crystals.

<table>
<thead>
<tr>
<th>Days</th>
<th>Control</th>
<th><em>H. indicus</em> 20 mg/5 ml</th>
<th><em>H. indicus</em> 10 mg/5 ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>90.37 ± 6.70</td>
<td>30.00 ± 2.49***</td>
<td>30.12 ± 2.98***</td>
</tr>
<tr>
<td>3.</td>
<td>204.44 ± 5.12</td>
<td>138.14 ± 2.91***</td>
<td>132.23 ± 3.47***</td>
</tr>
<tr>
<td>7.</td>
<td>312.00 ± 4.43</td>
<td>216.00 ± 2.54***</td>
<td>198.00 ± 3.00***</td>
</tr>
<tr>
<td>14.</td>
<td>222.00 ± 8.34</td>
<td>204.12 ± 3.49</td>
<td>180.00 ± 4.25**</td>
</tr>
<tr>
<td>21.</td>
<td>228.00 ± 6.86</td>
<td>204.00 ± 2.66*</td>
<td>222.24 ± 2.53</td>
</tr>
<tr>
<td>28.</td>
<td>294.00 ± 8.54</td>
<td>240.00 ± 3.84**</td>
<td>240.00 ± 3.00**</td>
</tr>
</tbody>
</table>

Sizes of crystals are given in microns. All values are mean ± SEM of six experiments in each set * P< 0.05 ; **P<0.01; ***P<0.001.
Fig. 3.14. Effect of aqueous extract of *H. indicus* root on the size of the calcium oxalate crystals.
Table 3.15

Effect of methanolic extract of *Parmelia perlata*

on the size of calcium oxalate crystals.

<table>
<thead>
<tr>
<th>Days</th>
<th>Control</th>
<th><em>P. perlata</em> 20 mg/5 ml</th>
<th><em>P. perlata</em> 10 mg/5 ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>48.67 ± 5.06</td>
<td>138.00 ± 6.58***</td>
<td>120.20 ± 7.43***</td>
</tr>
<tr>
<td>3</td>
<td>120.00 ± 6.87</td>
<td>180.56 ± 5.10***</td>
<td>144.00 ± 6.75</td>
</tr>
<tr>
<td>7</td>
<td>168.00 ± 4.45</td>
<td>198.00 ± 7.48*</td>
<td>156.40 ± 5.07</td>
</tr>
<tr>
<td>14</td>
<td>210.85 ± 6.58</td>
<td>168.00 ± 2.66**</td>
<td>168.00 ± 6.87**</td>
</tr>
<tr>
<td>21</td>
<td>174.00 ± 6.58</td>
<td>148.50 ± 3.44*</td>
<td>162.25 ± 3.36</td>
</tr>
<tr>
<td>28</td>
<td>180.00 ± 7.57</td>
<td>114.00 ± 3.96***</td>
<td>102.00 ± 2.95***</td>
</tr>
</tbody>
</table>

Sizes of crystals are given in microns. All values are mean ± SEM of six experiments in each set * P < 0.05; ** P < 0.01; *** P < 0.001
Fig. 3.15. Effect of methanolic extract of *P. perlata* on the size of the calcium oxalate crystals.
Table 3.16

Effect of aqueous extract of *Parmelia perlata* on the size of calcium oxalate crystals.

<table>
<thead>
<tr>
<th>Days</th>
<th>Control</th>
<th><em>P. perlata</em> 20 mg/5 ml</th>
<th><em>P. perlata</em> 10 mg/5 ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>90.37 ± 6.70</td>
<td>168.00 ± 5.17 ***</td>
<td>174.00 ± 2.80 ***</td>
</tr>
<tr>
<td>3</td>
<td>204.44 ± 5.12</td>
<td>268.14 ± 12.47 **</td>
<td>198.50 ± 5.03</td>
</tr>
<tr>
<td>7</td>
<td>312.00 ± 4.43</td>
<td>300.00 ± 6.72</td>
<td>312.00 ± 3.72</td>
</tr>
<tr>
<td>14</td>
<td>222.00 ± 8.34</td>
<td>216.56 ± 3.02</td>
<td>186.62 ± 4.31 *</td>
</tr>
<tr>
<td>21</td>
<td>228.00 ± 6.86</td>
<td>254.32 ± 3.64 *</td>
<td>252.00 ± 6.58</td>
</tr>
<tr>
<td>28</td>
<td>294.00 ± 8.54</td>
<td>282.00 ± 5.03</td>
<td>262.00 ± 3.43 *</td>
</tr>
</tbody>
</table>

Sizes of crystals are given in microns. All values are mean ± SEM of six experiments in each set * P< 0.05 ; **P<0.01; ***P<0.001.
Fig. 3.16. Effect of aqueous extract of *P. perlata*
on the size of the calcium oxalate crystals.
Table 3.17

Effect of methanolic extract of *Sida acuta* root on the size of calcium oxalate crystals

<table>
<thead>
<tr>
<th>Days</th>
<th>Control</th>
<th><em>S. acuta</em> 20 mg/5 ml</th>
<th><em>S. acuta</em> 10 mg/5 ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>48.67 ± 5.06</td>
<td>138.21 ± 6.92***</td>
<td>156.00 ± 7.32***</td>
</tr>
<tr>
<td>3.</td>
<td>120.00 ± 6.87</td>
<td>198.00 ± 7.71***</td>
<td>180.00 ± 6.58**</td>
</tr>
<tr>
<td>7.</td>
<td>168.00 ± 4.45</td>
<td>134.30 ± 2.50**</td>
<td>116.40 ± 2.91***</td>
</tr>
<tr>
<td>14.</td>
<td>210.85 ± 6.58</td>
<td>110.00 ± 5.07***</td>
<td>140.22 ± 3.34***</td>
</tr>
<tr>
<td>21.</td>
<td>174.00 ± 6.58</td>
<td>112.00 ± 4.25***</td>
<td>168.00 ± 3.50</td>
</tr>
<tr>
<td>28.</td>
<td>180.00 ± 7.57</td>
<td>96.12 ± 7.59***</td>
<td>162.00 ± 2.79</td>
</tr>
</tbody>
</table>

Sizes of crystals are given in microns. All values are mean ± SEM of six experiments in each set * P< 0.05; ** P< 0.01; *** P< 0.001.
Fig. 3.17. Effect of methanolic extract *S. acuta* root on the size of the calcium oxalate crystals.
Table 3.18

Effect of aqueous extract of *Sida acuta* root on the size of calcium oxalate crystals.

<table>
<thead>
<tr>
<th>Days</th>
<th>Control</th>
<th><em>S. acuta</em> 20 mg/5 ml</th>
<th><em>S. acuta</em> 10 mg/5 ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>90.37 ± 6.70</td>
<td>82.57 ± 3.20</td>
<td>84.50 ± 2.70</td>
</tr>
<tr>
<td>3.</td>
<td>204.44 ± 5.12</td>
<td>156.38 ± 3.18***</td>
<td>126.67 ± 2.53***</td>
</tr>
<tr>
<td>7.</td>
<td>312.00 ± 4.43</td>
<td>146.00 ± 2.09***</td>
<td>126.33 ± 2.91***</td>
</tr>
<tr>
<td>14.</td>
<td>222.00 ± 8.34</td>
<td>134.00 ± 3.45***</td>
<td>162.00 ± 3.02**</td>
</tr>
<tr>
<td>21.</td>
<td>228.00 ± 6.86</td>
<td>124.50 ± 3.32***</td>
<td>128.50 ± 2.30***</td>
</tr>
<tr>
<td>28.</td>
<td>294.00 ± 8.54</td>
<td>146.24 ± 4.70***</td>
<td>162.00 ± 3.41***</td>
</tr>
</tbody>
</table>

Sizes of crystals are given in microns. All values are mean ± SEM of six experiments in each set * P< 0.05 ; **P<0.01; ***P<0.001.
Fig. 3.18. Effect of aqueous extract of *S. acuta* root on the size of the calcium oxalate crystals
Table 3.19

Effect of methanolic extract of aerial parts of *Sida cordata* on the size of calcium oxalate crystals

<table>
<thead>
<tr>
<th>Days</th>
<th>Control</th>
<th><em>S. cordata</em> 20 mg/5 ml</th>
<th><em>S. cordata</em> 10 mg/5 ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>48.67 ± 5.06</td>
<td>90.50 ± 7.12**</td>
<td>90.00 ± 4.94**</td>
</tr>
<tr>
<td>3.</td>
<td>120.00 ± 6.87</td>
<td>138.50 ± 2.79</td>
<td>138.00 ± 5.12</td>
</tr>
<tr>
<td>7.</td>
<td>168.00 ± 4.45</td>
<td>180.17 ± 2.22</td>
<td>130.53 ± 2.49***</td>
</tr>
<tr>
<td>14.</td>
<td>210.85 ± 6.58</td>
<td>162.17 ± 7.00**</td>
<td>142.67 ± 5.93***</td>
</tr>
<tr>
<td>21.</td>
<td>174.00 ± 6.58</td>
<td>130.53 ± 5.07**</td>
<td>132.50 ± 5.77**</td>
</tr>
<tr>
<td>28.</td>
<td>180.00 ± 7.57</td>
<td>136.83 ± 4.94**</td>
<td>130.00 ± 3.55**</td>
</tr>
</tbody>
</table>

Sizes of crystals are given in microns. All values are mean ± SEM of six experiments in each set * P< 0.05 ; ** P< 0.01; *** P< 0.001.
Fig. 3.19. Effect of methanolic extract of aerial parts of *S. cordata* on the size of the calcium oxalate crystals.
Table 3.20

Effect of aqueous extract of aerial parts of *Sida cordata* on the size of calcium oxalate crystals

<table>
<thead>
<tr>
<th>Days</th>
<th>Control</th>
<th><em>S. cordata</em> 20mg/5ml</th>
<th><em>S. cordata</em> 10mg/5ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>90.37 ± 6.70</td>
<td>54.83 ± 2.79**</td>
<td>90.00 ± 8.39</td>
</tr>
<tr>
<td>3</td>
<td>204.44± 5.12</td>
<td>116.17 ± 1.66***</td>
<td>138.17 ± 4.94***</td>
</tr>
<tr>
<td>7</td>
<td>312.00± 4.43</td>
<td>156.47 ± 5.49***</td>
<td>194.47 ± 5.47***</td>
</tr>
<tr>
<td>14</td>
<td>222.00 ± 8.34</td>
<td>156.50 ± 2.61***</td>
<td>150.83 ± 2.61***</td>
</tr>
<tr>
<td>21</td>
<td>228.00 ± 6.86</td>
<td>162.00 ± 4.62***</td>
<td>176.83 ± 1.66***</td>
</tr>
<tr>
<td>28</td>
<td>294.00 ± 8.54</td>
<td>170.00 ± 4.31***</td>
<td>198.00 ± 4.30***</td>
</tr>
</tbody>
</table>

Size of crystals are given in microns. All values are mean ± SEM of six experiments in each set * P< 0.05 ; **P<0.01; ***P<0.001.
Fig. 3.20. Effect of aqueous extract of aerial parts of \textit{S. cordata} on the size of the calcium oxalate crystals.
extract added tubes were 172.4 μm (20 mg/5 ml) and 224 μm (10 mg/5 ml) on the 28th day.

The inhibitory effect of methanolic and aqueous extracts of *H. indicus* on the size of individual calcium oxalate crystals was studied at 20 mg/5 ml and 10 mg/5 ml concentrations and the results are presented in Table 3.13 and Table 3.14 and Fig. 3.13 and Fig. 3.14.

In the cases of methanolic extract of *H. indicus* statistically significant reduction in crystal growth could be found only on 14th to 28th day of crystal growth (Fig. 3.13). A significant increase in crystal growth was produced by both the concentrations on the first day and from 3rd to 7th day no marked change in crystal growth was observed for both the concentrations.

In the case of aqueous extract of *H. indicus* significant reductions in growth rate was observed on 1st, 3rd, 7th and 28th days for both the concentrations. On the 14th and 21st days the reduction in growth rate was less significant (Table 3.14. and Fig. 3.14.).

*Parmelia perlata* was tested for its effect on size of individual crystals at 20 mg/5 ml and 10 mg/5 ml levels. Table 3.15 and Fig. 3.15. show the effect of methanolic extract of *P. perlata* on the size of the crystals. The higher concentration of the drug elevated the crystal growth up to 7th day and thus it acted as a promoter till 7th day of crystal growth. From 14th day onwards significant reduction in growth rate was observed. The drug at 10 mg
level elevated the crystal growth up to the 3rd day and thereafter exhibited inhibitory property compared to that of the control.

The aqueous extract of *P. perlata* (Table 3.16 and Fig. 3.16) exhibited enhancing effect on crystal growth at both the concentration on several days of the experiment. Inhibitory effect was observed only on 14th and 28th day of the experiment where the inhibition was not significant for higher concentration (20 mg/5 ml).

The methanolic extract of *Sida acuta* at 20 mg/5 ml and 10 mg/5 ml levels retarded the growth of individual crystals from 7th day onwards. On statistical evaluation, the growth reduction by the drug was found to be highly significant. The inhibitory effect was better for higher concentration on 14th, 21st and 28th days. The results are presented in Table 3.17 and Fig. 3.17.

The aqueous extract of *Sida acuta* at both 20 mg/5 ml and 10 mg/5 ml levels exhibited highly significant reductions in the growth of individual calcium oxalate crystals. Size of the crystals in both the extract treated tubes were more or less same on 1st to 28th days, but far less than that in the control tubes. The stagnation in growth is evident from Table 3.18 and Fig. 3.18.

The methanolic extract of *S. cordata* at both 20 mg/5 ml and 10 mg/5 ml levels elevated the growth of individual CaOX crystals for the first few days, thereafter the drug retarded the growth. The growth rate was almost same in both the concentration. The reduction in growth rate from 14th to 28th day was statistically significant. Table 3.19 and Fig. 3.19 explain these effects.
It is evident that 10 mg of S. cordata could reduce crystal growth from 7th day onwards and 20mg of the drug from 14th day onwards.

The inhibitory effect of aqueous extract of S. cordata on the size of individual CaOX crystals is depicted in Table 3.20 and Fig. 3.20. On analysis of the data it can be seen that the drug at both levels show identical effect, but quantitatively the effect by 20 mg was higher than that by 10 mg of the drug. The reduction in growth rate is also highly statistically significant.

3.5.3 Infrared spectroscopy

The calcium oxalate crystal grown in-vitro on adding ethanol / distilled water (control) and the ethanolic solution of the methanolic extract / aqueous extract of medicinally important parts of Dichrostachys cinerea, Hemidesmus indicus, Parmelia perlata, Sida acuta and Sida cordata were subjected to Infrared Analysis. The analysis confirmed the presence of calcium oxalate monohydrate crystals and the results are presented in Fig. 3.21 to Fig. 3.32.

The crystals grown in the present investigation was characterized by infrared spectroscopy using JASCO FT–IR–410 by KBr pellet method. The IR spectra of grown calcium oxalate monohydrate (COM) crystals are shown in the Fig. 3.21 – 3.32. The band at 517 cm\(^{-1}\) arises due to O–C–O in–plane bending. The bands at 662 and 781cm\(^{-1}\) are due to the out–of–plane O–H bending and C–H bending mode respectively. The band at 885 cm\(^{-1}\) was assigned to C–C stretching mode. The C–O stretching and O–H bending is
Fig. 3.21.

Fig. 3.22.

Fig. 3.23.

Fig. 3.24.

Fig. 3.25.

Fig. 3.26.
Fig. 3.27.

Fig. 3.28.

Fig. 3.29.

Fig. 3.30.

Fig. 3.31.

Fig. 3.32.
Fig. 3.21. IR spectra of calcium oxalate crystals grown in the presence of ethanol (control)

Fig. 3.22. IR spectra of calcium oxalate crystals grown in the presence of distilled water (control)

Fig. 3.23. IR spectra of calcium oxalate crystals grown in the presence of methanolic extract of *D. cinerea*

Fig. 3.24. IR spectra of calcium oxalate crystals grown in the presence of aqueous extract of *D. cinerea*

Fig. 3.25. IR spectra of calcium oxalate crystals grown in the presence of methanolic extract of *H. indicus*

Fig. 3.26. IR spectra of calcium oxalate crystals grown in the presence of aqueous extract of *H. indicus*

Fig. 3.27. IR spectra of calcium oxalate crystals grown in the presence of methanolic extract of *P. perlata*

Fig. 3.28. IR spectra of calcium oxalate crystals grown in the presence of aqueous extract of *P. perlata*

Fig. 3.29. IR spectra of calcium oxalate crystals grown in the presence of methanolic extract of *S. acuta*

Fig. 3.30. IR spectra of calcium oxalate crystals grown in the presence of aqueous extract of *S. acuta*

Fig. 3.31. IR spectra of calcium oxalate crystals grown in the presence of methanolic extract of *S. cordata*

Fig. 3.32. IR spectra of calcium oxalate crystals grown in the presence of aqueous extract of *S. cordata*
observed at 1316 cm$^{-1}$. The broad band at 1617 cm$^{-1}$ was assigned to C–O asymmetric stretch. A group of four bands occurring at 3062, 3256, 3338 and 3490 cm$^{-1}$ are due to symmetric and asymmetric O–H stretch, which are the characteristic IR spectral bands of COM.$^{112-114}$

There was no change in the absorption maxima in the samples studied. The IR spectra of CaOX crystals from the control set up (Fig. 3.21 and Fig. 3.22) and IR spectra of CaOX crystals from the extract set up (Fig. 3.23–3.32) were compared and it was found that none of the IR spectra differed from that of the control indicating no possibility of any type of chemical bonding between the drug and the calcium oxalate crystals.

3.5.4 Scanning electron microscopy

Calcium oxalate crystals grown in the control set up and that grown in the presence of methanolic/aqueous extracts of the medicinally important parts of *Dichrostachys cinerea*, *Hemidesmus indicus*, *Parmelia perlata*, *Sida acuta* and *Sida cordata* were subjected to SEM studies for understanding the structural changes if any by the various extracts. Fig. 3.33 show the SEM of *in–vitro* grown calcium oxalate crystals from the control set up viz., crystal grown in the presence of ethanol. Crystals from control experiments contained individual prismatic crystals (Fig. 3.33a), twinned crystals (Fig. 3.33b) and some aggregates (Fig. 3.33c).

Addition of methanolic extract of *D. cinerea* resulted in the formation of more single crystals and, agglomeration was less when compared to that of
the control set up. Most of the crystals were deformed and showed well defined long cracks. There was a tendency for the crystals to break into bits at the cracked sites (Fig. 3.34).

Crystals grown with methanolic extract of *H. indicus* showed less defined edges, cracks, crevices and dots of corrosion which could mean a tendency to reduce the size by crushing the stones into small bits (Fig. 3.35).

Methanolic extract of *P. perlata* when included in the crystal growth set up resulted in crystal deformation (Fig. 3.36).

Crystals from methanolic extract of *S. acuta* set up (Fig. 3.37) contained some deformed and smaller crystal aggregates in addition to single and twinned crystals. Additions of the extract resulted in partial dissolution of the crystal with cracks and crevices and a tendency for chipping away of the parts as compared to control.

Methanolic extract of *S. cordata* could change the shape of the crystals with less number of aggregates. The crystals also showed tendency for chipping away of parts (Fig. 3.38).

Fig. 3.39 show the SEM of calcium oxalate crystals grown in the presence of distilled water. The sample contained single prismatic crystals (Fig. 3.39a), twinned prismatic crystals (Fig. 3.39b) and bunched prismatic crystals (Fig. 3.39c).
Fig. 3.34. SEM of calcium oxalate crystals on adding ethanolic solution of the methanolic extract of *D. cinerea* (a) showing deformation (b) showing cracks and withering away of parts.

Fig. 3.35. SEM of calcium oxalate crystals on adding ethanolic solution of the methanolic extract of *H. indicus* (a) showing single and twinned crystals (b) showing less defined edges.
Fig. 3.36. SEM of calcium oxalate crystals on adding ethanolic solution of the methanolic extract of *P. perlata* (a) showing deformed crystals (b) showing irregular surface

Fig. 3.37. SEM of calcium oxalate crystals on adding ethanolic solution of the methanolic extract of *S. acuta* (a) showing single and twinned crystals (b) showing tendency for chipping away of the parts.
Fig. 3.38. SEM of calcium oxalate crystals on adding ethanolic solution of the methanolic extract of *S. cordata* (a) showing less
Fig. 3.39. SEM of calcium oxalate crystals on adding distilled water
(a) showing individual prismatic crystal
(b) showing twinned crystal  (c) showing crystal aggregate.
Aggregation of crystals could rarely be seen when aqueous extract of *D. cinerea* was introduced in the crystal growth set up (Fig. 3.40). Most of the crystals were single crystals and had cracked edges. This could indicate a dissolution effect for the extract.

As in the previous case, addition of aqueous extract of *H. indicus* also resulted in an increase in the number of single crystals and decreased the tendency of agglomerate formation (Fig. 3.41).

Aqueous extract of *P. perlata* showed more crystal aggregates and rosette–shaped crystals could be seen (Fig. 3.42).

Aqueous extract of *S. acuta* showed a marked reduction in the tendency to form crystal aggregates as is evident from (Fig. 3.43). Aqueous extracts when included in the crystal growth set up also resulted in corrosion, crevices in the crystal which could indicate a dissolution effect for the drug.

Aqueous extract of *S. cordata* when included in the crystal growth set up resulted in more aggregation. The crystals were seen adherent to each other. Most of the crystals were deformed with irregular surfaces (Fig. 3.44).

Thus crystals grown in the presence of different extracts showed difference in number and morphology. Methanolic and aqueous extracts of *D. cinerea*, aqueous extract of *H. indicus* and *S. acuta* were effective in inhibiting crystal aggregation where as aqueous extracts of *P. perlata* and *S. cordata* promoted crystal aggregation. Most of the extracts could produce deformed crystals with irregular surfaces and shapes.
Fig. 3.40. SEM of calcium oxalate crystals on adding aqueous extract of *D. cinerea* (a) showing single crystals (b) showing cracked edges.

Fig. 3.41. SEM of calcium oxalate crystals on adding aqueous extract of *H. indicus* (a) showing mostly single crystals (b) showing cracked edges.
Fig. 3.42. SEM of calcium oxalate crystals on adding aqueous extract of *P. perlata* (a) showing rosette shaped crystals  
(b) showing more aggregation

Fig. 3.43. SEM of calcium oxalate crystals on adding aqueous extract of *S. acuta* (a) showing reduction in the tendency to form crystal aggregates  
(b) showing corrosion
Fig. 3.44. SEM of calcium oxalate crystals on adding aqueous extract of *S. cordata* (a) showing aggregation (b) showing crystal deformation
3.6 DISCUSSION

Urinary stones are a common cause of morbidity, and recurrent urinary stone disease can cause chronic morbidity and ultimate death from renal failure. The majority, particularly the common calcium oxalate-containing stones, are due to the additive interaction of multiple environmental and, as yet incompletely understood, genetic factors. Of the several approaches to the study of urinary calculus aetiologies, it is only the analysis of calculi themselves which enables investigators to accurately characterize the chemical conditions prevailing at the time of nucleation and growth. The stones are more often than not multi-component systems containing crystalline and non-crystalline material which in turn can be organic and/or inorganic in composition. In addition, many different morphologies, sometimes of the same chemical species are observed.\(^{115}\)

Several physico-chemical techniques have been investigated and applied by Rodger\(^{115}\) in the qualitative and quantitative analysis of over 400 urinary and other human calculi. These techniques include X-ray powder diffraction [XRD], Infrared spectroscopy [IR], Scanning Electron Microscopy [SEM] in conjunction with an energy dispersive X-ray analyzer, Transmission Electron Microscopy [TEM], X-ray Fluorescence spectrometry [XRF], Atomic Absorption Spectrophotometry [AAS], Density Gradient Analysis [DGA], Thermo Gravimetric Analysis [TGA], Chemical Analysis [CA] and Inductively Coupled Plasma Atomic Emission Spectroscopy [ICPAES]. All have been
shown to possess inherent advantages on the one hand and limitations on the other. The so called "finger print methods", XRD and IR, can identify stone constituents with a certainty that cannot be matched by other techniques, but they too have their limitations. SEM and TEM permit morphological studies at the ultrastructural level and can yield meaningful data concerning stone initiation and growth mechanisms. However, conclusions based on morphology alone are risky.\textsuperscript{115}

Calcium oxalate crystal nucleation, growth and agglomeration in urine are believed to be controlled by a balance between the ambient physico-chemical super saturation and inhibitors capable of acting on one or more of these crystallization steps. Grover\textsuperscript{116} et al have studied the inhibition of growth and aggregation of calcium oxalate crystals \textit{in-vitro} on the basis of the average size of particles crystallized and also by scanning electron microscopy. If inhibitors of crystal formation were not able to act and control their size, the final result will be nephrolithiasis and / or nephrocalcinosis. Crystalluria with oxalate crystal volume measurement is a non-invasive, easily performed investigation and can give feedback on the efficacy of urolithiasis therapy.\textsuperscript{117,118}

Systematic studies on the growth of urinary crystals will be useful to derive information about the mechanism of formation of urinary stones.\textsuperscript{107} Studies on the growth of urinary crystals in the presence of some substances
which act as inhibitors or promoters of crystal growth may help to pick out potential substances for use by recurrent stone formers.

In the crystals growth experiments, the following inhibitory effect may be observed in general\(^{107}\): (i) no nucleation (giving only a powdery mass), (ii) reduction in the number and size of the crystals, (iii) reduction in the total mass of the crystals formed, (iv) change in the morphology of the crystals and (v) change in the crystalline quality (good to poor quality). The promotery effects that may be observed are the following: (a) increase in the number and size of the crystals and (b) increase in the total mass of the crystals formed.

By carefully observing the shape, size, transparency and approximate number of crystals obtained and also from the knowledge of lengths of crystal columns conclusion were derived regarding the inhibitory or promotery effect of the extracts incorporated.

### 3.6.1 Lengths of crystal columns

Results presented in Table 3.1 to Table 3.10 and Fig. 3.1 to Fig. 3.10 summarise the effects of methanolic and aqueous extracts of all the five medicinal plants on calcium oxalate crystal growth \textit{in–vitro}.

The results clearly show that all the five plant drugs, tested are effective in reducing the lengths of crystal columns. Among the methanolic extracts tested, \textit{Sida acuta} is the most effective in reducing the lengths of crystal columns followed by \textit{Dichrostachys cinerea} and \textit{Parmelia perlata} is
the least effective in reducing the lengths of crystal columns. Concentration independent inhibitory effect was observed for the methanolic extract of *D. cinerea* and *Sida cordata* with regard to the lengths of crystal columns, where the lengths of crystal columns were almost equal for higher and lower concentrations. Concentration dependent inhibitory effect was observed for other methanolic extracts where the degree of inhibition was more with higher concentration.

Among the various aqueous extracts tested, only *D. cinerea* was effective in reducing the lengths of crystal columns where as for other extracts, the reductions in the lengths of crystal columns were statistically less significant.

3.6.2 Size of individual crystals

It was observed that all the extract tested exhibited inhibitory effect to a large or smaller extent in the growth of individual crystals. The results are provided in Table 3.11 to Table 3.20 and Fig. 3.11 to Fig. 3.20. Among the methanolic extracts, *P. perlata* and *S. acuta* were more effective in reducing the size of individual crystals. Among the aqueous extracts, *S. acuta, S. cordata* and *D. cinerea* at 20 mg level were more effective in reducing the size of the individual crystals.
3.6.3 Infrared Spectroscopy (IR)

When infrared light is passed through a sample of an organic compound, some of the frequencies are absorbed, while other frequencies are transmitted through the sample without being absorbed. If we plot absorbance or transmittance against frequency, the result is an infrared spectrum.\textsuperscript{119} Infrared spectra contain many absorptions associated with the complex interacting vibrating systems in the molecule, and this pattern of vibrations, since it is uniquely characteristic of each molecule, gives rise to a uniquely characteristic set of absorption bands in the spectrum. This band pattern serves as a fingerprint of the molecule.\textsuperscript{119}

Infrared spectroscopy has been used as an easy and accurate method for the identification of specimens by determining molecular structure. Infrared analysis is more specific and reproducible than usual wet chemical methods.\textsuperscript{120} IR spectroscopy has been successfully carried out for the quantitative and semi-quantitative analysis of urinary calculi. Identification of the unknown sample is done by comparing its spectrum with reference spectra according to the procedure of Hesse and Bach.\textsuperscript{121} Compared to other techniques, only a very small quantity of the material is required for IR analysis. Simplicity of operation of the IR technique is an added advantage.\textsuperscript{115}

The extract treated crystals exhibit IR spectra (Fig. 3.23 to Fig. 3.32), similar to that of the control (Fig. 3.21 and 3.22) and this rules out any
possibility of binding between the drug and calcium oxalate crystals. There was no change in the absorption maxima in the IR spectra of samples studied when the extracts were introduced in the crystal growth set up and none of the IR spectra differed from that of control indicating no possibility of any type of chemical bonding between the drug and the calcium oxalate crystals.

3.6.4 Scanning electron microscopy (SEM)

Identification of the constituents present in a urinary calculus often provides the basis for the clinical management of urolithiasis in recurrent stone formers and is of primary importance in the investigation of stone aetiology. In the very first instance, SEM permits the identification of urinary (and other) stone constituents on the basis of crystal shape and chemical composition.\textsuperscript{122}

Scanning electron microscopy is used for the identification of microstructural characteristics of solid objects and is more advantageous due to the high resolution and three dimensional appearance of the object. The greater depth of focus of SEM enables us to have more information about the sample.

By means of SEM analysis Hesse \textit{et al.}\textsuperscript{123} have demonstrated characteristic calcium oxalate and calcium phosphate crystals in urinary stones.
All samples of non conducting materials for SEM study have to be given a thin coating of conducting material. Usually gold – palladium (60:40) metal coating is given by the popular method of sputtering.\textsuperscript{124}

SEM is a highly recommended, easy-to-apply procedure which, when coupled with one of the finger print methods (XRD or IR), can provide much useful data concerning stone growth patterns.\textsuperscript{125,126} Furthermore, when SEM morphology of the stones was tested for its accuracy in the analysis of 100 consecutive urinary stones, more than 95% of common stone components were identified by those who had no previous experience in this field but who had received only a few hours of training. The data indicated further a strong potential of the SEM as a tool for the analysis of urinary stones.\textsuperscript{127}

Fig. 3.33 to Fig. 3.44. given above indicated the SEM of \textit{in-vitro} grown calcium oxalate crystals in silica gel medium. Calcium oxalate monohydrate crystal has a well defined prismatic shape. Addition of the extracts resulted in partial dissolution of the crystals at the edges in most of the cases. Methanolic and aqueous extracts of \textit{D. cinerea} (Fig. 3.34 and Fig. 3.40), aqueous extracts of \textit{H. indicus} (Fig. 3.41) and \textit{S. acuta} (Fig. 3.43) were effective in inhibiting crystal aggregation whereas aqueous extracts of \textit{P. perlata} and \textit{S. cordata} promoted crystal aggregation (Fig. 3.42 and Fig. 3.44). Agglomeration of calcium oxalate crystals plays a major role in urinary stone formation and has the potential to produce very large crystals in a short period of time with no reduction of supersaturation.\textsuperscript{128} There are two different kinds of
agglomeration: i) primary and ii) secondary agglomeration. Primary agglomerations is the growth of crystals on the surface or the tips of the crystals already formed. Secondary agglomeration is that resulting from crystal to crystal collision. Primary agglomeration was recognized as a possible mechanism for the development of a calculi.\textsuperscript{129} The conditions prevailing in the kidney are not conducive to the secondary agglomeration process and is expected to play only a minor role in the formation of stones. Natarajan\textsuperscript{107} et al. have reported that change in morphology and change in the crystalline quality may be considered as inhibitory effects in crystal growth experiments. In the present investigation the morphology of crystals have changed in a few cases. In the tubes containing methanolic extract of \textit{P. perlata} (Fig. 3.36.) most of the crystals were deformed with irregular shape. Platy crystals were obtained (Fig. 3.37) when methanolic extract of \textit{S. acuta} was introduced in the crystal growth set up.

Calcium oxalate crystals grown in the presence of most of the extracts showed pictures with cracks, withering away of parts, crevices and dots of corrosion which could mean a tendency to reduce the size by crushing the stones into small bits.
3.7 CONCLUSION

Growing crystals is a slow and careful process because the crystals grow by adding single layers of molecules. The crystal shape reflects the basic patterns by which the molecules of the crystal build up. In the present study calcium oxalate crystals were grown in the presence of methanolic and aqueous extracts of five medicinal plants. Observations of the crystals under optical and scanning electron microscopes revealed single, twinned and bunched prismatic morphologies. All the extracts tested are effective in inhibiting the crystal growth \textit{in–vitro} at both levels. Methanolic and aqueous extracts of \textit{D. cinerea}, aqueous extracts of \textit{H. indicus} and \textit{S. acuta} were effective in inhibiting crystal aggregation. Methanolic extracts of \textit{P. perlata} and \textit{S. acuta} could produce deformed crystals with irregular shape and surfaces. All the methanolic extracts are effective in reducing the lengths of crystal columns and also the size of individual crystals. All the aqueous extracts tested are effective in reducing the size of individual crystals. There findings justify the usefulness of all the five medicinal plants in the treatment of urinary disorder by Ayurvedic medical practioners.
3.8 EXPERIMENTAL

3.8.1 Collection and preparation of plant extracts

*Parmelia perlata* was collected from Thantrikudi, Dindigul District of Tamil Nadu and *Dichrostachys cinerea, Hemidesmus indicus, Sida acuta* and *Sida cordata* were collected respectively from Thirukurunkudi, Thenmalai, Tirunelveli and Shenkotta of Tirunelveli District of Tamil Nadu, India in the month of September. The plant materials were identified by Dr. V. Chelladurai, Research Officer (Botany), Survey of Medicinal and Aromatic plant Unit – Siddha, CCRAS, Palayam Kottai, Tirunelveli District, Tamil Nadu, India and voucher specimens have been deposited at the Department of Chemistry, Manonmaniam Sundaranar University, Tirunelveli District, Tamil Nadu, India [*Dichrostachys cinerea* (MSU 051), *Hemidesmus indicus* (MSU 052), *Parmelia perlata* (MSU 053), *Sida acuta* (MSU 054) and *Sida cordata* (MSU 055)].

The medicinally important parts were removed and washed thoroughly with water, cut into small pieces, dried under shade for two weeks and powdered. The powdered plant materials were individually and successively extracted with petroleum ether (40°–60°C), benzene, chloroform, methanol and water. The last trace of the solvent was removed under reduced pressure distillation and the crude extract was dried in a vacuum desiccator and used for the experiments. Aqueous extract was kept in a refrigerator in a tightly closed, clean container. Dried methanolic and aqueous extracts were weighed exactly and dissolved respectively in ethanol and distilled water so
as to get solutions of two different concentrations, 20 mg/5 ml and 10 mg/5 ml. These different concentrations were prepared on the basis of quantity of plant extract in 100 ml solvents and the actual concentration of the active ingredients were not taken into consideration.

3.8.2 *In-vitro* crystal growth

3.8.2.1 Single diffusion method

Twenty milliliters of sodium metasilicate solution of density 1.03 g/ml was taken and the pH adjusted to 6.3 using 3M acetic acid. To this 5ml of 1M calcium chloride was added, mixed and set aside in Hane's tube for gel formation. After gellation, 5 ml of 1M oxalic acid was slowly added over the gel followed by 5 ml of ethanol/water as the supernatant solution for control group and 5 ml of ethanolic solution of dried methanolic extract/aqueous extract of the test plant for other groups.105

3.8.2.2 Effect of methanolic and aqueous extracts on calcium oxalate growth *in-vitro*

Calcium oxalate crystals were grown *in-vitro* in silica gel medium in Hane's tubes by single diffusion method according to the procedure mentioned above.105 For testing an extract of each plant three sets of Hane's tubes, each set consisting of 6 tubes were arranged. One set served as the control where 5 ml of ethanol / distilled water was added as the supernatant liquid. In second set 5 ml of methanolic / aqueous extract of the test plant of concentration 20 mg/5 ml was added and in the third set 5 ml of methanolic /
aqueous extract of the test plant of concentration 10 mg/5 ml was added. The experiment was performed at room temperature (27° ± 3°C). The crystals appeared as cloudy precipitate and in due course, the depth of the column was found to increase. The thickness (length) of the crystal columns were measured in centimeter on days 1, 3, 7, 10, 15, 20, 25 and 30. Size and morphology of the crystals were noted on days 1, 3, 7, 14, 21 and 28 and the reading of the test groups were compared with the readings of the control. For microscopic examination, the crystals were pipetted out from the gel medium, placed on a glass slide, sticking gel was homogenized gently with distilled water, a cover glass was placed on top and viewed. A calipered micrometer was made use of for measuring the size of the crystals. After day 30, the crystals were cleared off the gel by repeated washing with water, filtered through a filter paper and air dried. The purity and morphology of the crystals thus obtained were assessed by infrared spectroscopy and scanning electron microscopy.

3.8.3 Statistical analysis

The results are analysed statistically and Student’s ‘t’ test\textsuperscript{130} employed for calculating P values. Levels up to 0.05 have been considered significant. All values are mean ± SE of six samples.

3.8.4 Infrared Spectroscopy

The instrument used in this study was an infrared spectrophotometer JASCO FT-IR-410 which could measure in the wave length region
4000–200 cm\(^{-1}\). Potassium bromide (KBr) which does not have any absorption in the spectral region was used as the inert carrier. 1 mg of the powered material for analysis was homogenized with 200 mg of KBr. This mixture was pressed into a pellet under vacuum to exclude moisture, using a normal pressing machine and fed into the IR spectroscope for analysis. The evaluation of the IR spectrum was done by comparison with reference spectra.

### 3.8.5 Scanning Electron Microscopy

Dry specimen is used for SEM analysis. The specimen mounting brass stud was cleaned, and small particles of the sample to be analysed were glued to the stud using silver paste, a conductive adhesive. The non-conductive samples were made conductive by sputtering them with gold to an approximate thickness of 100 Å. A gold sputtering unit was used for the purpose.\(^\text{124}\)

A JEOL JSM 35-C scanning electron microscope was used for the study. The gold sputtered samples were placed in the vacuum chamber of the instrument and viewed. The signals emitted from the surface of the specimen due to the hitting of the electron beam, were taken up by the detector, amplified and picked up by the cathode ray tube of the display screen on which the image is obtained. Relevant fields were photographed. Reading of photographs was done based on the results of the qualitative and quantitative estimations, IR Spectroscopy as well as by comparing with reference photographs.\(^\text{123}\)
3.9 REFERENCES


