Chapter VI

Growth Inhibition Study of Struvite

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6.1 Introduction

This chapter describes the growth inhibition study of struvite crystals. Struvite type urinary calculi can grow rapidly forming "staghorn-calculi", which is more painful urological disorder. It has also high degree of recurrence. As mentioned earlier in chapter II, struvite stones are among the most difficult and dangerous problems in stone disease because of the potential of life-threatening complications from infection. Epidemiological studies from various countries continue to report a frequency of the occurrence of struvite stones of between 25 % and 38 % [1,2]. Many surgical options are available as medical management options for struvite calculi. As described earlier in section 2.10 of chapter II, surgical options may include extracorporeal shock wave lithotripsy, ureteroscopic stone extraction and percutaneous nephrolithotomy. Recurrence is the core issue in the clinical management of struvite calculi. Although, surgical management has become increasingly tolerable, medical prevention of recurrent struvite calculi is feasible, easily obtained and greatly desirable. In such a condition, it is the need of society to discover such drugs, which will inhibit struvite growth, in addition to high success rates, excellent safety profile, low side effect profile, and ease of use, such a drug will be ideal for management of calculi. Therefore, it is of prime importance to study the growth and inhibition of struvite crystals in vitro. In the present investigation in vitro single diffusion gel growth technique was used to study the growth and inhibition behavior of struvite crystals by using the herbal extracts like Boerhaavia diffusa Linn, Commiphora wightii and Rotula aquatica Lour as well as the fruit juice of Citrus medica Linn. The goal of this growth inhibition study was dual: (i) to find inhibition efficiency of these herbal extracts and fruit juice
on struvite crystal growth as well as to provide a perspective for further investigation of their possible use in stone therapy, and (ii) to identify versatile and “green” inhibitors of struvite.

6.2 Brief Review of Inhibition Studies of Urinary Calculi

There are substances, which may change or modify urinary crystal formation, can be divided into three main groups: (i) Inhibitors, (ii) Promoters, and (iii) Complexors. Substances that reduce the crystallization are called inhibitors and contrary to this, which increase crystallization are termed as promoters. The details of the inhibitor and promoters are already discussed in section 2.6.6 of chapter II. Urinary inhibitors attach to the growth sites on crystalline face and retard the growth and aggregation further.

In the literature one can find information on glutamic acid, α-ketoglutaric acid [3], magnesium, ethylene diamine tetra acetic acid (EDTA), citrate [4,5], phytic acid [6], pyrophosphate [5,7], diphosphonate [7], amino acids like lysine [8], aspartic acid [4], ornithine, tryptophan [9], organic acids such as tartaric, malonic, citric, hippuric [10,11] and osteopontin (OPN) [4], which have kidney stone dissolving properties.

Many chemicals are found to be having inhibitive actions on the growth of urinary stones and crystals; for example, magnesium, citrate, pyrophosphate and nephrocalcin are the common inhibitors for calcium phosphate crystals [12], tartrates were found as a good inhibitor in natural and artificial urine media [13], some amino acids, which show the influence on the spontaneous precipitation of calcium oxalate and depend on the type and the concentration of amino acids used [14], the citrate compounds, which show the inhibition of nucleation and growth steps of COM crystallization [15], some
phosphate derivatives, such as, D fructose-1.6-diphosphate, pyrophosphate methylene diphosphonate and phytate are found to be strongly inhibiting the growth of COM seed crystallization [16]. Inhibition of calcium oxalate crystal formation is exhibited by citrate, pyrophosphate, glycosaminoglycans, RNA fragments and nephrocalcin [17,18]. Moreover, the inhibition of COM aggregation is possible by mainly two glycoproteins, namely, nephrocalcin and Tamm-Horsfall glycoprotein [19]. Certain substances that form soluble complexes with lattice ions for specific crystals and decrease the free ion activity of that particular ion and as a result effectively decrease the state of saturation for that ion system. It has been found that both citrate and magnesium not only act as inhibitors but also as complexors [20,21]. Sometimes, a substance may promote one stage of crystal formation such as growth and inhibit another stage such as aggregation, for example, glycosaminoglycans promote crystal nucleation but inhibit crystal aggregation and growth [22].

Many researchers have reported the growth inhibition study of various urinary type crystals as follows:

**Inhibition of calcium oxalate**: Several researchers have concentrated growth inhibition studies on calcium oxalate due to its highest epidemiological rates. Atanassova et al [3,8] showed the inhibiting effect of α-ketoglutaric acid and DL-lysine on calcium oxalates. Inhibition and dissolution of calcium oxalate crystals in solutions containing a homoeopathic medicine *Berberis Vulgaris*-Q, amino acids such as aspartic acid, glutamic acid, α-keto glutaric acid, a naturally occurring inhibitor, and juices of some fruits of citrus group such as lemon and orange were reported by Das et al [23]. Moreover, Das et
al [24] reported the use of extracts of the edible plant _Trianthema monogyna_ (commonly known as pathari, ghetuli, vasu or lana sata) a naturally growing herb of north eastern Uttar Pradesh, India, and the pulse _Macrottyloma uniflorum_ (commonly known as kurthi or horsegram) in the inhibition / dissolution of calcium oxalate and gallbladder stone. Qiu et al [25] reported that citrate modified the shape and inhibited the growth of COM crystals by selectively pinning step motions on the (-101) face, whereas leaving the (010) to grow uninhibited. 

Recently, _in vitro_ inhibition studies of COM crystal growth using various Mediterranean traditional Algerian medicinal plants such as _Ammodaucus leucotrichus, Ajuga iva, Erica multiflora_ and _Stipa tenacissima, Globularia alypum, Atriplex halimus, Tetraclinis articulata, Chamaerops humilis_ and _Erica arborea_ were carried out by M. Beghalia et al [26] and found the extracts of these plants as potent inhibitors of COM. Among these, the two plant extracts that exhibited highest potency on calcium oxalate crystallisation, _Ammodaucus leucotrichus_ was found to inhibit potently the nucleation, growth, and aggregation phases of crystallization, but _Erica multiflora_ inhibited nucleation and growth of the crystals but not their aggregation [27]. Recently, Frackowiak et al [28] reported that the extract of _Humulus lupulus_ L. have high potency for inhibition and dissolving synthetic calcium oxalate crystals as well as real kidney stones, obtained from patients after surgery. Farook et al [29] have estimated the inhibition efficiency of medicinal plants _Achyranthes aspera_ Linn, _Passiflora leschenaultii_ DC, _Solena amplexicaulis_ (Lam.) Gandhi, _Scoparia dulcis_ Linn and _Aerva lanata_ (Linn.) on the mineralization of calcium oxalate, calcium carbonate and calcium phosphate. Aal et al [30] indicated the
inhibition of calcium oxalate by extract of *Ammi visnaga*. Bensatal and Ouharani [31] showed inhibition of crystallization of calcium oxalate by the extraction of *Tamarix gallica* L. Joshi et al [32] studied *in vitro* growth inhibition of COM crystals by herbal extracts of *Tribulus terrestris* Linn., and *Bergenia ligulata* Linn.

**Inhibition of Brushite**: *In vitro* growth inhibition study on brushite crystals in the presence of citric acid and lemon juice along with the artificial reference urine and natural urine was reported by Joshi and Joshi [33], which showed strong inhibition of growth of brushite crystal. Later on Joshi et al [34] reported the inhibition of the growth of brushite crystals using aqueous extracts of *Tribulus terrestris* Linn and *Bergenia ligulata* Linn, whereas Joseph et al [35] reported the inhibitory effect of tartaric acid and tamarind on brushite.

**Inhibition of Hydroxyapatite**: Parekh et al [36] reported the growth inhibition of hydroxyapatite crystals at physiological temperature *in vitro* by using herbal extracts of *Boswellia serrata* Roxb., *Tribulus terrestris* Linn, *Rotula aquatica* Lour, *Boerhaavia diffusa* Linn and *Commiphora wightii* herbal extract solutions and the diffusion constants were measured for Ca$^{2+}$ ions.

**Inhibition of Cystine**: D. Heimbach et al [37] reported that the combination of 2% acetylcysteine with alkaline solutions, especially tris-hydroxymethylene-aminomethane (THAM) at a pH of 10.0, is an effective tool in the management of cystine stone disease.

**Inhibition of Calcium Phosphates**: Recently, Das and Verma [38] reported the inhibitory effect of grape extract on the tri-calcium phosphate. Parekh [39] conducted *in vitro* growth inhibition experiments for calcium pyrophosphate
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tetrahydrate crystals using herbal extracts of *Rotula aquatica* Lour, *Boerhaavia diffusa* Linn, and *Commiphora wightii*.

**Inhibition of Struvite**: Growth inhibition reports on struvite crystals are scarce in literature. Earlier, *in vitro* inhibition of struvite crystals grown by *Proteus mirabilis* in artificial urine in the presence of acetohydroxamic acid (AHA) was reported by Downey et al [40]. The AHA primarily acts as a urease inhibitor, which may disrupt the struvite growth and formation directly through the interference with the molecular growth processes on crystal surface. Phosphocitrate was reported to inhibit *in vivo* formation of struvite [41]. Crystal growth studies and molecular modeling results indicate strong affinity of phosphorocitrate to (1 0 1) faces of struvite [42]. Natarajan et al [43] carried out crystal growth experiments of some urinary crystals by incorporating the extracts or juices of some natural products in the gel media to find their inhibitory or promotery effects.

**6.3 Gel Growth – A Simplified In Vitro Model**

The growth of crystals in static solution is generally explained with the help of standard laws of physical chemistry. However, the normal urine in a human body is not a static solution, but new solutes are added and subtracted from the solution. Moreover, it is difficult to mimic the urinary tract *in vitro*; however, the growth of crystals in a gel medium under static environment helps explain the growth of urinary calculi in a body to a certain extent. Hence the growth of urinary calculi can be simulated in a laboratory by growing crystals in a silica hydro gel medium. The gel framework, though chemically inert, provides a three dimensional matrix in which the crystal nuclei are delicately held and provides a substratum for a gradual supply of nutrients for
growth. Slow and controlled diffusion of reactants in gels can mimic the condition in a body. Bio-crystallization usually occurs in the slow and steady process in the soft tissues, cavities or vessels. Growth of crystals with different morphologies is commonly found in bio-crystallization. In the gel growth technique, by changing the growth conditions, crystals with different morphologies and sizes can be obtained. The main advantage is that the crystals can be observed practically in all stages of their growth. The crystal growth by gel method provides simulation of synovial cartilage and other biological fluids [35,44]. The growth of urinary crystals in silica hydro gel can be considered as a simplified in vitro model of the highly complex growth of urinary calculi in vivo.

The in vitro growth inhibition or dissolution study is important as the growth of calculi continues to occur with the supply of nutrients through urine and the inhibition process has to be achieved. In the gel growth, nutrients are constantly supplied to the growing crystals and the dissolution or inhibition is to be checked for the selected solutions.

The growth of crystals from the gel, the simplest technique under ambient conditions, is suitable for the growth of bio-materials crystals, urinary type crystals and certain other compound crystals, which are sparingly soluble and decomposes at low temperatures or decomposes before melting. The gel density, pH and concentration of the reactants are important factors influencing the growth of good quality single crystals at room temperatures. This technique has been elaborately explained in Chapter-III. This technique has been employed as a simple technique to grow various urinary type crystals [45-48] and study the role of various inhibitors in vitro [33,35].
Recently, a modified gel growth technique has been proposed for the micro-crystal growth and in situ observations, which has been successfully tested for brushite micro-crystal growth inhibition in the presence of citric acid [49].

6.4 Medicinal Plants Used for Growth Inhibition Studies

The ancient Indian treatise written in Sanskrit like Rig Veda, Atharva Veda (4500-1600 BC), Ayurveda (sub section of Atharva Veda), Charaka Samhita (approximately 1500 BC), Sushruta Samhita (600 BC) and Ashtanga Hridaya Samhita (approximately 700 AD) mention the use of several plants as medicine. In the indigenous Indian system of medicine i.e., in the Ayurveda, many herbal medicines have been recommended for the treatment of urinary stone problem and some of them have been experimentally evaluated [24,29,33-36,43,50].

6.4.1 Boerhaavia diffusa Linn

Nomenclature: Boerhaavia diffusa Linn is a medicinal plant commonly known as Punarnava, Raktapunarnava, Raktakanda, Raktapushpa, Kshudra, Varshaketu, Varshabhu or Shothaghni in Sanskrit [51,52], Spreading Hogweed in English. It has different names in different Indian languages – Biskhapra in Hindi; Gadhapurma in Bengali; Satodi, Dholia-saturdo, Motosatoda in Gujarati; Thzhuthama in Malayalam; Mukaratte in Tamil and Itsit in Punjabi. It is also known as Huang Xi Xin or Huang Shou Dan in China, Pigweed in USA, Erva Tostao in Brazil. The plant was named by Linnaeus in honour of Hermann Boerhaave, a famous Dutch physician of the 18th century [53]. The meaning of diffusa in the name is "spreading". The meaning of the name Punarnava in Sanskrit is “Punah punarnava bhawati iti” which is translated as that which becomes fresh again and again. The plant is known
as *Punarnava* due to two reasons: (i) The name probably derived from the perennial habit of the plant, which remains dry and dormant during summer and regenerates from the same old root stock in the rainy season [54], and (ii) The name Punarnava may derived from the Sanskrit phrase “Karoti shariram punarnavam” that denotes such therapeutic property which translates as “that which rejuvenates the body”.

**Plant Description:** *Boerhaavia diffusa* Linn (*B. diffusa*) is a herbaceous plant of the family Nyctaginaceae. It is a perennial diffuse herb with stout root stock and many branches. *B. diffusa* is up to 1 m long or more, having spreading branches. The stem is prostrate, woody or succulent, cylindrical, often purplish, hairy, and thickened at its nodes. Leaves are simple, green, thick, fleshy, hairy and arranged in unequal pairs. Leaves are ovate-oblong, acute or obtuse, rounded or subcordate at base, glabrous above, whitish beneath. Flowers pale rose in color, in irregular clusters of terminal panicles. Fruits highly viscid, easily detachable, one seeded, indehiscent with thin pericarp. It has a large root system bearing rootlets. The tap root is tuberous, cylindrical to narrowly fusiform, conical or tapering, light yellow, brown or brownish grey. It is thick, fleshy and very bitter in taste.

![Boerhaavia Diffusa Linn Plant, Leaves, Flowers, Fruits, Roots](Photo: Hugh Wilson)

*Figure: 6.1 Boerhaavia Diffusa* Linn Plant, Leaves, Flowers, Fruits, Roots [55, 56]
**Distribution**: *B. diffusa* is found in the tropical, subtropical and temperate regions of the world. It is distributed in India, China, Australia, Pakistan, Egypt, Sudan, Sri Lanka, USA, South Africa and in several countries of the Middle East [57].

**Chemical Composition**: The plant extract contains a large number of biochemical compounds as listed in table 6.1.

**Table 6.1: Biochemical Compounds Present in *Boerhaavia Diffusa* Linn [58-68]**

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<th>Group</th>
<th>Biochemical Compounds Present</th>
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<tbody>
<tr>
<td>Alkaloids</td>
<td>Punarnavine 1-2, hypoxanthine 0-L-arabinofuranoside</td>
<td>[58-60]</td>
</tr>
<tr>
<td>Flavonoides</td>
<td>Boeravino A to F, Flavone, 5-7-dihydroxy-3’-4’-dimethoxy-6-8-dimethyl</td>
<td>[61]</td>
</tr>
<tr>
<td>Xanthone</td>
<td>Borhavine</td>
<td>[62]</td>
</tr>
<tr>
<td>Steroids</td>
<td>β-sitosterols, Campesterol, Daucosterol, Sitosterol oleate, Sitosterol palmitate, Stigmasterol</td>
<td>[63]</td>
</tr>
<tr>
<td>Triterpenoides</td>
<td>Ursolic acid</td>
<td>[64]</td>
</tr>
<tr>
<td>Lipids – Organic acids</td>
<td>Arachidic acid, Behenic acid, Glycerol, Heptadecyclic acid, Oleaic acid, Palmitic acid, Stearic acid, Tetracosanoic, Hexacosanoic</td>
<td>[65]</td>
</tr>
<tr>
<td>Carbohydrates</td>
<td>Fructose, Galactose, Glucose, Sucrose, Xylose</td>
<td></td>
</tr>
<tr>
<td>Proteids</td>
<td>Alanine, Aspartic acid, Glutamic acid, Glutamine, Glycoproteins, Histidine, Leucine, Methionine, Proline, Serine, Threonine, Tyrosine, Valine</td>
<td></td>
</tr>
<tr>
<td>Alkane</td>
<td>Triacontan-1-ol, Oxalic acid; Hentriacontane, n</td>
<td></td>
</tr>
<tr>
<td>Lignans</td>
<td>Liriodendrin, Syringaresinol-mono-beta-d-glucoside</td>
<td>[66]</td>
</tr>
<tr>
<td>Others</td>
<td>Punarnavoside, Boerhaavic acid, Potassium nitrate</td>
<td>[67,68]</td>
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**Medicinal Properties**: The whole plant or its specific parts (leaves, stem, and roots) are known to have medicinal properties. In Ayurveda the plant is used for the treatment of many diseases [69-74], such as diabetes, stress, dyspepsia, abdominal pain, inflammation, jaundice, hepatitis, gastro-intestinal disorders (as laxative) enlargement of spleen, congestive heart failure and bacterial infections. The plant is known to possess anti-inflammatory [75,76],
anticonvulsant [77-80], antifibrinolytic [66], diuretic [81-84], hepatoprotective [85-88], antidiabetic [89,90] and immunomodulatory [91-93] activities. It was also reported to be useful in the treatment of elephantiasis, night blindness, corneal ulcers and nephritic syndrome [94-96]. Olaleye et al [97] reported antioxidant and hepatoprotective properties of the extracts of *B. diffusa* leaves in pharmacological models. Punarnavine, an alkaloid isolated from *B. diffusa* has been shown *in vitro* anticancer [98], antiestrogenic [99] and immunomodulatory [100] activity.

### 6.4.2 Commiphora wightii

**Nomenclature**: The name is derived from Greek words ‘kommis’ and ‘phora’ meaning gum bearer. In Indian languages, it is known by various names like gugal in Gujarati, guggul in Hindi, guggulu in Sanskrit, gukkulu and maishakshi in Tamil, Indian bdellium in English, *Mo ku er mo yao* in Chinese. Detailed descriptions regarding the actions, uses, and indications as well as the varieties of *C. wightii* have been described in the Ayurvedic treatises.

![Figure: 6.2 Commiphora Wightii Plant, Leaves, Flower, Fruit, Stem, Gum](image)

**Plant Description**: *C. wightii* is a flowering plant in the family Burseraceae. It is a shrub or small tree, 1.5–2 m in height, reaching a maximum height of 4 m, with thin papery bark [102]. It has a thick main stem with short, thorny
branches. The leaves are non-hairy, simple or trifoliate and the leaflets ovate. Leaves have aromatic smell. It is a slow growing, endangered medicinal tree [103,104]. Flowers are small, brownish red, occurring singly or in groups of 2-3. Fruit is an ovoid green berry like drupe, reddish and 6-8 mm in diameter. It provides yellowish oleo gum resin (commonly known as gum guggul) which is extracted from the bark by a process called tapping. A plant generally takes 10 years to reach tapping maturity under the prevailing dry climatic conditions.

**Distribution**: The tree is found in rocky and open hilly areas or rough terrain and sandy tracts in warm and semiarid to arid areas. It is distributed in India, Nepal, Srilanka, China, Bangladesh and Pakistan. In India it is found in arid, rocky tracts of Rajasthan (Thar Desert), Aravalli range, Gujarat (Kachchh and Saurashtra regions) [105], Maharashtra, Madhya Pradesh, Karnataka and Eastern Himalayas [106]. The plant may be found from northern Africa to central Asia, but is most common in northern India.

**Chemical Composition**: Many groups have studied the phytochemistry of *C. wightii* and found ferulates [107], steroids [108], guggulsterones [109] guggutetrols [110]. New antifungal flavanone, muscanone was isolated along with known naringenin from *C. wightii* by Fatope et al [111]. The golden yellow oleo-gum-resin is a complex mixture of over two dozen ketones, several phenolics, diterperoids, flavonoids and sterols [112,113].

*C. wightii* contains Guggulsterol-I $\{C_{27}H_{44}O_{4}\}$, Guggulsterol-II $\{C_{27}H_{46}O_{3}\}$, Guggulsterol-III $\{C_{27}H_{44}O_{3}\}$, Guggulsterol-IV $\{C_{27}H_{44}O_{3}\}$, Guggulsterol-V $\{C_{29}H_{50}O_{4}\}$, Guggulsterol-VI $\{C_{21}H_{32}O_{2}\}$. The active components of the plant are the guggulsterones specifically the
stereoisomers, guggulsterone-E \( \text{C}_{21}\text{H}_{28}\text{O}_2 \), and guggulsterone-Z \( \text{C}_{21}\text{H}_{28}\text{O}_2 \), which are potent lipid and cholesterol lowering natural agents \[114,115\].

**Medicinal Properties** : *C. wightii* is one of the most important medicinal plants used in the herbal system of medicine. A monograph of all the major citations of its use was published by Apte \[116\]. *C. wightii* is used as an Ayurvedic medicine for the treatment of joint pains, arthritis, hyperlipidemia, inflammation, sciatica, paralysis, convulsions, gonorrhea, chronic cough and cold, bronchitis, diabetes, urinary disorder, dysuria, calculi, fever, skin ailments, stress, ulcers, cardiovascular disease, lipid disorders, high cholesterol \[117,118\], obesity and other weight-related problems. *C. wightii* is effective as a weight-loss and fat burning agent. It increases white blood cell counts and possesses strong disinfecting properties. Various studies to check most of these therapeutic effects of *C. wightii* were carried out by different researchers \[72,119-122\]. The guggul resin has proven medicinal properties and is used to cure various diseases such as hypercholesterolemia \[123\], cardiovascular diseases \[124\] and cancerous diseases \[125\]. The cardiovascular therapeutic benefits of guggul and guggulsterone appear to be due to the multiple pharmacological activities, notably the hypolipidemic, anti-oxidant, and anti-inflammatory effects \[124\]. The main ingredients of *C. wightii* are flavonoids which have potent anti-oxidant actions \[111\]. It has also shown dose dependent anti-inflammatory activity and was also found to control inflammation and pain in osteoarthritis patients \[126\]. Recently Ishnava \[127\] et al reported antibacterial potential of gum resin of *C. wightii*. 

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6.4.3 *Rotula aquatica* Lour

**Nomenclature**: *Rotula aquatica* Lour (*R. aquatica*) is commonly known as *Pashanbheda* or *Ashmahabheda* in Sanskrit and *Lung guan mu* in Chinese.

**Plant Description**: *R. aquatica* belonging to the family boraginaceae, is a rheophytic woody aromatic medicinal shrub. Generally, reaches a height of 2 to 3 m. The leaves are alternate, imbricate, ovate-oblong, usually 0.8 to 1.5 cm in length and short-stalked. The flowers are small, crowded and pink.

*Figure: 6.3 Rotula Aquatica Lour Plant, Flower and Root [128]*

**Distribution**: *R. aquatica* is distributed in India, Sri Lanka, China, tropical southeastern Asia including Indonesia, Malaysia, Myanmar, Philippines, Thailand, Vietnam, Africa, Brazil and Latin America [129]. The plant is scattered throughout peninsular and eastern India in the sandy and rocky beds of streams and rivers.

**Chemical Composition**: The sterol, rhabdiol, polyphenols (tannins), glycosides and ureide allantoin were isolated from the roots by researchers [50,129-131]. The plant contains Baunerol [132], steroid and alkaloids [131].

**Medicinal Properties**: Usually, plant root is used for medicinal purpose. It is used for treatment of cough, cardiac disorder, blood disorders, fever, ulcers, poisons, dysuria, bladder stone, cancer, piles and venereal diseases in Ayurveda [72, 133-137]. In Ayurveda, it is a well-known lithotriptic (stone-
dissolving) drug [138]. In India *R. aquatica* is one of the most extensively used medicinal plants to dissolve urinary calculi. The root tuber of *R. aquatica* is astringent, bitter, diuretic, cooling, laxative and also lithotriptic. A decoction of root is diuretic and used for treating stones in bladder [139]. The aqueous extract of the root of *R. aquatica* showed antioxidant and antiurolithiatic activity. Sterol and rhabdiol were found to be active to induce diuresis (division or separation of a structure's parts) and allantoin is responsible for diuretic activity [130]. The antiurolithiatic activity of the root extract can be attributed to its diuretic activity. The aqueous extract of *R. aquatica* root has shown crystal dissolving activity against monosodium urate monohydrate type urinary calculi [50], whereas ethyl acetate extract of *R. aquatica* root showed significant antilithic activity against struvite and calcium oxalate stones [140]. The stems were also used in diuretic decoctions and found effective in preventing cell proliferation of pancreatic cancer cell lines [131]. Christina et al demonstrated stone inhibitory effect of *R. aquatica* root decoction in male Wistar rats [141]. Recently, Mengi et al [142] investigated anti-inflammatory potential of aqueous extract of *R. aquatica* roots in acute and chronic inflammatory conditions in rats. Moreover, acute toxicity studies revealed that *R. aquatica* root extract was found safe at all doses when administered orally to rats, up to a dose of 2000 mg kg$^{-1}$. *R. aquatica* is well known for the treatment of urinary calculi, but its inhibitory property on struvite has not been scientifically reported previously. Therefore, in the present study the inhibitory property of the aqueous extract of *R. aquatica* root on struvite has investigated.
6.4.4 *Citrus Medica* Linn

**Nomenclature**: In the present growth inhibition study the author has used one of the citrus fruits - *Citrus medica* Linn., commonly known as *Baranimbu*, *Bijaura* or *Bijoru* in Hindi, *Matulunga* in Sanskrit, Citron in English, *Cidro* in Spanish, *Zitronatzitrone* in German, *Fo shou* in Chinese and as *Bushukan* in Japanese. The designation *medica*, given it by Linnaeus, is apparently derived from its ancient name "Median or Persian apple". The general descriptions, characteristics, constituents, various medicinal use were discussed by many researchers [143,144].

**Plant Description**: *Citrus Medica* Linn is belonging to the Rutaceae family. It is an evergreen armed shrub with straggling thorny branches and smooth yellowish brown bark. Generally, this shrub reaches a height of 2.4 to 4.5 m. Leaves are oblong or elliptic with acute or rounded apex, coriaceous (leathery; stiff and tough, but somewhat flexible), glabrous (without surface ornamentation such as hairs, scales or bristles), pellucid-punctate (tiny marked with dots in leaves visible when held in front of light), dull dark green and lemon-scented. Leaflets are 7.5 - 15 cm long. Flowers are white tinged with pink and scented. The fruit is fragrant, mostly oblong, obovoid or oval. The size of the fruit varies greatly from 9 to 30 cm. Peel is usually very thick, rough and bumpy, which becomes yellow when ripe. It contains numerous seeds.

**Distribution**: It grows in evergreen forests naturally or can also be cultivated. Citrus is grown in tropical and subtropical regions of the world and occupies a wide range of latitude over which it is being cultivated. The north-eastern Indian states are rich treasure of various citrus species and their varieties. It is
found in the base region of Himalaya from Gadwal to Sikkim. It is also seen in Assam, Central India and Western Ghats of India. It is now cultivated commercially in the Mediterranean region and, to a lesser extent, in the West Indies, Florida, and California.

Figure : 6.4 Citrus Medica Linn Plant, Leaves, Flowers, Fruits

**Chemical Composition** : The fruit juice contains good amount of citric acid - \( \{C_6H_8O_7\} \) along with ascorbic acid \( \{C_6H_8O_6\} \), hespiridin \( \{C_{28}H_{34}O_{15}\} \), campesterol \( \{C_{28}H_{48}O\} \), stigmasterol \( \{C_{29}H_{48}O\} \), \( \beta \)-sitosterol \( \{C_{29}H_{50}O\} \), malic acid \( \{C_4H_6O_5\} \), phosphoric acid \( \{H_3PO_4\} \), potassium citrate \( \{C_6H_5K_3O_7\} \), mucilage and sugar [145]. The peels are reported to contain coumarins- \( \{C_9H_6O_2\} \), limettin \( \{C_{11}H_{10}O_4\} \), scoparone \( \{C_{11}H_{10}O_4\} \), scopoletin \( \{C_{10}H_{9}O_4\} \) and umbelliferone \( \{C_9H_6O_3\} \) [137]. Seeds of the fruit contain limonin \( \{C_{26}H_{30}O_8\} \), limonol \( \{C_{26}H_{32}O_8\} \) and nomilinic acid [146].

**Medicinal Properties** : Various parts of this plant are widely used in Indian traditional system of medicine [69,147-149]. Ripe fruits are used in sore throat, cough, asthma, arthritis, rheumatism, thirst, hiccough, ear ache and vomiting. It is potent anti-scorbutic, stomachic, tonic, stimulant, expellant of poison, correct fetid breath; distilled water of the fruit is sedative; fruits and
seeds are cardiac tonic and useful in palpitation. Fruit juice also acts in stimulating liver for proper secretion of bile juices. Roots, flowers, seeds, peels and leaves are also used in many ailments. Fruit extracts have also shown good antioxidant activity [150]. Recently, it is reported that the analgesic activity of fruit decoction [151] and antimicrobial activity of fruit juice as well as juiceless fruit pulp extract [152] of Citrus medica Linn are noteworthy.

6.5 Preparation of Herbal Extracts

6.5.1 Plants Material: B. diffusa roots obtained from Vasai, Thane district, Maharashtra and the R. aquatica Lour, roots were collected from Savantwadi, Maharashtra in the Winter season and authenticated by Dr. M. R. Almeida, Taxonomist, Mumbai. C. wightii gum resin was obtained from Barda, Gujarat in Spring and authenticated by Dr. P. S. Nagar, Saurastra University, Rajkot.

6.5.2 Extraction: The respective plant materials were cleaned, dried, powdered (50 g) and added to distilled water (400 mL). The mixture was heated in a boiling water bath until it reduced to half of the original volume. The extract was dried in a rotary vacuum evaporator to a syrupy consistency and then in a steam bath to thick, pasty consistency. The extracts were stored in glass vials kept in airtight plastic boxes in refrigerator and used to investigate inhibitory effect on struvite type urinary calculi. All the extracts were prepared at Bhavan’s SPARC at Mumbai and were provided to the present author for the study. Two different concentrations, namely 0.5% and 1.0% of aqueous root extracts of B. diffusa and R. aquatica as well as aqueous gum resin extract of C. wightii were used to determine the inhibitory effect.
6.6 Growth Inhibition Study of Struvite by Herbal Extracts

6.6.1 Single Diffusion Gel Growth Technique

The single diffusion gel growth technique was used to study the growth and inhibition behavior of struvite crystals in the presence of different herbal extracts. Sodium Metasilicate (SMS)-\( \text{Na}_2\text{SiO}_3.9\text{H}_2\text{O} \) solution of specific gravity 1.05 was used to prepare the gel. An aqueous solution of Ammonium Dihydrogen Phosphate (ADP)-\( \text{NH}_4\text{H}_2\text{PO}_4.2\text{H}_2\text{O} \) of 0.5 M concentration was mixed with the SMS solution in appropriate amount so that the pH value 7.0 could be set for the mixture. The gel solution of 20 mL was transferred into the test tubes of 140 mm length and 25 mm diameter. All test tubes and other glassware were autoclaved at 120°C for 15 min. Here, the silica gel was chosen because it remains stable and does not react with the reacting solutions or with the product crystal formed. After the gelation took place, 20 mL supernatant solutions (SS) of pure (i.e. control solution, without inhibitor) 1.0 M magnesium acetate-\( \text{C}_4\text{H}_6\text{MgO}_4.4\text{H}_2\text{O} \) and 1.0 M magnesium acetate prepared with 0.5 % and 1.0 % concentrations of the each of the herbal extracts were gently poured on the set gels in test tubes. This was done in the aseptic medium in a laminar flow hood to avoid microbial contaminations. Composition and the pH value of the SS are as shown in table 6.2. After pouring SS, the test tubes were capped with airtight stopples. The experiment was conducted at the room temperature.

The following reaction is expected to occur in the gel between the two reactants:

\[
\text{NH}_4\text{H}_2\text{PO}_4.2\text{H}_2\text{O} + (\text{CH}_3\text{COO})_2\text{Mg.4H}_2\text{O} \rightarrow \text{NH}_4\text{MgPO}_4.6\text{H}_2\text{O} + 2\text{CH}_3\text{COOH} \quad (6.1)
\]
The apparent lengths of growing/dissolving struvite crystals in each of the test tubes were measured by using a traveling microscope of least count 0.001 cm at regular time interval. The apparent lengths of growing/dissolving struvite crystals at different depth from the gel-liquid interface in each of the test tubes were measured and mean length of the crystals at different depth was calculated. The statistical analysis of the single factor ANOVA was carried out. The total mass and total volume of struvite crystals in each test tubes were measured after removal of crystals and per test tube the yield of crystals was obtained for each concentrations with and without herbal extracts.

### 6.6.2 Struvite Crystals Grown in the Gel Media

The gel-grown struvite crystals exhibit different morphologies, viz. dendritic, prismatic, rectangular platelet and needle type depending upon the location of growth. As shown in figure 6.5, at gel-liquid interface, dendritic-type crystals were observed, whereas at higher depths in the gel from gel-liquid interface, prismatic-type crystals were observed. Due to higher concentrations
of reactants at the gel – liquid interface, more-or-less, a direct reaction took place, which might resulted in to dendritic type crystals.

![Figure 6.5](image1.jpg)

Figure 6.5 (a) Dendritic crystals grown at the gel-liquid interface
(b) Prismatic crystal grown in the gel at higher depths

![Figure 6.6](image2.jpg)

Figure 6.6 Struvite Crystals Grown in the Gel Medium
[(a) No Inhibitor, (b) 0.5 % B. Diffusa Linn, (c) 1.0 % B. Diffusa Linn, (d) 0.5 % C. Wightii, (e) 1.0 % C. Wightii, (f) 0.5 % R. Aquatica Lour, (g) 1.0 % R. Aquatica Lour]

It was noticed that the number of grown struvite crystals and their average apparent length in the silica-hydro gel medium decreased with the increasing concentrations of the extracts in the SS. The reduction in the number density of the grown struvite crystals in the test tubes with extracts proved the inhibitory effect of the herbal extracts experimented. It was found that the crystals grown in the test tubes without the extract were transparent to translucent diaphaneity. On the other hand, some of the crystals grown in the test tubes with the *R. aquatica* extract showed dark brown colorization which might be due to inclusion of the extract in the crystals. Figure 6.6 shows the struvite crystals grown in the gel medium for different extracts.
6.6.3 Growth of Struvite Crystals at Gel – Liquid Interface

After pouring of the SS, dendritic type struvite crystals were found to grow at the gel–liquid interface. The growth rates of crystals, at the end of first, second, third and fourth day, growing in the gel at the gel–liquid interface for different concentration of SS are as presented in table 6.3. It can be noticed from the table that the growth rates are comparatively lower for each of the herbal extracts, whereas they are comparatively higher for the control solution without inhibitor. Moreover, the growth rates are decreased with the increasing concentration of each extract. It is also noticed that the growth rates decrease with time in each concentration. The lowest growth rate 0.638 cm/day is observed for the SS with 1.0 % R. aquatica, while the highest growth rate 1.185 cm/day is noticed for control solution. The average apparent lengths of the dendritic crystals growing in the gel at gel-liquid interface are increased up to first 4 days for control solution, whereas they are increased just for first 2 days in the both concentrations of B. diffusa; for 3 days in 0.5 % C. wightii, only for 1 day in 1.0 % C. wightii; for 2 and 1 day in 0.5% and 1% R. aquatica extract solutions, respectively, and followed by dissolution.

Table : 6.3 : Growth of Struvite Crystals at Gel-Liquid Interface

<table>
<thead>
<tr>
<th>Growth Rate (cm/day)</th>
<th>Day</th>
<th>No Inhibitor (Control Solution)</th>
<th>Concentration of Herbal Extract</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>B. Diffusa Linn</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>0.5 %</td>
</tr>
<tr>
<td>Growth Rate (cm/day)</td>
<td>1</td>
<td>1.185</td>
<td>1.027</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>0.608</td>
<td>0.534</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>0.410</td>
<td>D. S.*</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>0.309</td>
<td>-</td>
</tr>
</tbody>
</table>

* D. S. = Dissolution Started
Here, the lower values of growth rate in comparison to control solution, the reduction in growth rates with the increasing concentrations of each tested extracts as well as the reduction in growth rates with the time proved the inhibitory effect of the extracts.

![Graph showing the maximum length of dendritic type struvite crystals grown at the gel-liquid interface in different concentration](image)

**Figure : 6.7 Maximum length of Dendritic Type Struvite Crystals Grown at the Gel-Liquid Interface in Different Concentration**

The histogram in figure 6.7 shows the maximum apparent lengths of the dendritic type struvite crystals at the gel – liquid interface in each concentration. It is clear from the figure that the apparent lengths of the crystals are remarkably inferior in all the cases with herbal extracts in comparison to control solution. It can also be noticed that the apparent lengths are decreased with the increasing concentration of extracts.

The extent of inhibition of growing struvite crystals at the gel – liquid interface at the end of first day are tabulated in table 6.4. Interestingly, the significant percentage of inhibition is noticed for every herbal extract studied. The maximum percentage of inhibition 46.16 % is observed for 1.0 % *R. aquatica* extract, where as minimum 13.33 % inhibition is observed for 0.5 % of *B. diffusa* extract.
Table 6.4: Inhibition of Struvite Crystal Growth in Gel at Gel–Liquid Interface in the Presence of Different Herbal Extracts on the First Day

<table>
<thead>
<tr>
<th>Apparent Crystal Length (cm)</th>
<th>No Inhibitor (Control Solution)</th>
<th>Concentration of Herbal Extract</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>B. Diffusa Linn 0.5 % 1.0 %</td>
<td>C. Wightii 0.5 % 1.0 %</td>
</tr>
<tr>
<td></td>
<td>R. Aquatica Lour 0.5 % 1.0 %</td>
<td></td>
</tr>
<tr>
<td>Average Length</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.185</td>
<td>1.027</td>
<td>0.825</td>
</tr>
<tr>
<td>0.740</td>
<td>0.715</td>
<td>0.745</td>
</tr>
<tr>
<td>0.638</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Reduction in Length</td>
<td>-</td>
<td>0.158</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.360</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.445</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.470</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.440</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.547</td>
</tr>
<tr>
<td>% of Inhibition</td>
<td>-</td>
<td>13.33%</td>
</tr>
<tr>
<td></td>
<td></td>
<td>30.37%</td>
</tr>
<tr>
<td></td>
<td></td>
<td>37.55%</td>
</tr>
<tr>
<td></td>
<td></td>
<td>39.66%</td>
</tr>
<tr>
<td></td>
<td></td>
<td>37.13%</td>
</tr>
<tr>
<td></td>
<td></td>
<td>46.16%</td>
</tr>
</tbody>
</table>

6.6.4 Growth and Dissolution of Struvite Crystals at Gel–Liquid Interface

The growth and dissolution of dendritic type struvite crystals at gel-liquid interface is shown by plots of average length versus time period in figure 6.8.

Figure 6.8 Growth and Dissolution of Struvite Crystals at Gel-Liquid Interface

It is observed that struvite crystals grown at gel – liquid interface also dissolved to some extent even in the absence of herbal extract. It may be due to partial dissolution of the crystals in acetic acid produced as a byproduct of the chemical reaction as shown in equation (6.1), which forms the compound for crystals growth. But, at the same time the dissolution rates are enhanced in all the concentrations of herbal extracts. Table 6.5 depicts the dissolution
rates, enhanced dissolution rates as well as the percentage of enhanced dissolution rates due to the presence of herbal extracts.

Table 6.5: Dissolution Rate of Struvite Crystals at Gel-Liquid Interface

<table>
<thead>
<tr>
<th>Concentration of Herbal Extract</th>
<th>No Inhibitor (Control Solution)</th>
<th>B. Diffusa Linn</th>
<th>C. Wightii</th>
<th>R. Aquatica Lour</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.5 %</td>
<td>0.5 %</td>
<td>0.5 %</td>
<td>0.5 %</td>
</tr>
<tr>
<td></td>
<td>1.0 %</td>
<td>1.0 %</td>
<td>1.0 %</td>
<td>1.0 %</td>
</tr>
<tr>
<td>Dissolution Rate</td>
<td>1.8 x 10^{-2}</td>
<td>3.4 x 10^{-2}</td>
<td>4.3 x 10^{-2}</td>
<td>2.4 x 10^{-2}</td>
</tr>
<tr>
<td>Enhanced Dissolution Rate</td>
<td>-</td>
<td>1.6 x 10^{-2}</td>
<td>2.5 x 10^{-2}</td>
<td>0.6 x 10^{-2}</td>
</tr>
<tr>
<td>% of Enhanced Dissolution Rate</td>
<td>-</td>
<td>88.89%</td>
<td>138.8%</td>
<td>33.33%</td>
</tr>
<tr>
<td>Days</td>
<td>33</td>
<td>21</td>
<td>40</td>
<td>24</td>
</tr>
<tr>
<td>Number of Days Required for Complete Dissolution of the Dendritic Type Struvite Crystals Grown at the Gel – Liquid Interface</td>
<td>28</td>
<td>33</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Here, the dissolution rates for all herbal extracts are remarkably higher than that of control solution. Moreover, dissolution rates are significantly increased with increasing the concentration of herbal extract, except for R. aquatica. Enhanced dissolution rates are observed for all the herbal extracts. It is clear from the table that the maximum percentage of enhanced dissolution rate 138.8% is observed in the case of 1.0% B. diffusa.

The number of days required for the complete dissolution of the dendritic type struvite crystals grown at the gel – liquid interface are also mentioned in table 6.5, which shows that minimum 21 days are required for the complete dissolution of crystals grown at gel – liquid interface for the concentration of SS with 1.0% B. diffusa.

From the analysis of tables 6.4 and 6.5, it can be perceived that the extracts of R. aquatica have retarded the growth rate from the very beginning.
of the crystal growth, whereas extracts of \textit{B. diffusa} have speed up the dissolution rates once the growth of the crystals took place. Here, the percentage of enhanced dissolution rates in all the tested concentrations of herbal extracts confirmed the inhibitory effect of the extracts.

6.6.5 Depth of Growth of Struvite Crystals in Gel Column

The depth of growth, i.e., the depth from the gel-liquid interface in gel column up to which struvite crystals are growing, versus time period plots are shown in figure 6.9 for all concentrations of herbal extracts selected for study.

![Figure: 6.9 Depth of Growth During First Four Days After Pouring of SS](image)

From the figure it is clear that the maximum depth of growth 5.2 cm is attained in just 2 days after the pouring of SS in case of no inhibitor (control solution), where as the maximum depths of growth after 2 days are restricted to just 2.7 and 2.4 cm for 0.5 % and 1.0% concentrations of \textit{C. wightii} extracts, respectively; 2.9 cm and 2.6 cm for 0.5 % and 1.0 % concentrations of \textit{B. diffusa} extracts, respectively; while 3.0 cm and 2.8 cm for 0.5 % and 1.0 % concentrations of the \textit{R. aquatica} extracts, respectively. This suggests that the extracts impede the diffusion process of reactants occurring in the gel column for the nucleation and, subsequently, the growth of crystals. Thus, the
reduction in depths of growth indicates the inhibition offered by all the three herbal extracts.

6.6.6 Growth and Dissolution of Struvite Crystals at Different Depth

Growth and dissolution of struvite crystals at different depth in gel from the gel–liquid interface in the absence of herbal extract is shown by the plots of average length versus time period in figure 6.10.

![Figure: Growth of Struvite Crystals at Different Depth from the Gel-Liquid Interface in the Absence of Inhibitor](image)

From this figure one can notice that the average length of growing crystals in the gel at gel–liquid interface increases up to first 4 days and then it decreases by indication of dissolution, due to the formation of acetic acid. It is noticed that the average length of growing crystals at different depth from the gel–liquid interface increases up to first 7 days and then it remains constant. As the depth of the gel column increases, the average size of the grown crystals is found to be gradually smaller.

The growth and dissolution of struvite crystals at different depths in the gel column from the gel – liquid interface in case of 0.5 % and 1.0 % concentrations of *B. diffusa*, *C. wightii*, and *R. aquatica* are studied and the
plots of average length of crystals versus time period are shown in figures 6.11 to 6.13, respectively.

Figure : 6.11 Growth and Dissolution of Struvite at Different Depth from the Gel-Liquid Interface for 0.5% *B. Diffusa* Linn (Left) and 1.0% *B. Diffusa* Linn (Right)

Figure : 6.12 Growth and Dissolution of Struvite at Different Depth from the Gel-Liquid Interface for 0.5% *C. Wightii* (Left) and 1.0% *C. Wightii* (Right)

Figure : 6.13 Growth and Dissolution of Struvite at Different Depth from the Gel-Liquid Interface for 0.5% *R. Aquatica* (Left) and 1.0% *R. Aquatica* (Right)

From the above figures it is clear that the similar phenomena are observed more effectively in the presence of all the tested herbal extracts. It was
noticed that the growth rate as well as the apparent size of the crystals grown were lower, which also proved the inhibitory effect of the extracts.

6.6.7 Variation in the size of Prismatic Type Struvite Crystals

Figure 6.14 shows the dimension of the grown prismatic type struvite crystals in all the tested concentrations of SS with and without herbal extracts.

![Figure 6.14 Variation in the Size of Prismatic Type Struvite Crystals](image)

From the figure it can be perceived that the average dimension of the prismatic type crystals grown with the herbal extracts are comparatively smaller than that of control solution, which also gives an idea of the inhibitory effect of all the three evaluated extracts. The least average dimension of prismatic crystals is found to be 0.1 cm for the 1.0 % *C. wightii* extract.

6.6.8 Fragmentation of Struvite Crystals

The phenomenon of fragmentation or fracture of the grown struvite crystals due to the presence of the herbal extract was quite interesting and deserved further attention. The incorporation of extract not only allowed the crystalline face to grow further, but presumably weakened the existing bonds, leading to cracking and further fracture into fragments. The depth of fragmentation of grown crystals, i.e., the depth from the gel-liquid interface up
to which the crystals start breaking near the gel-liquid interface was noticed for each of the concentrations of the assessed herbal extracts. Figure 6.15 shows the plots of the depth of fragmentation versus time interval. Initially, the concentration of extracts in the gel column was less and gradually built up due to diffusion into the gel from the SS, which consequently increased the depth of the fragmentation with the passage of time.

![Figure 6.15 Depth of Fragmentation versus Days](image)

It was also observed that at higher depths some of the fragmented crystals retained their critical size. As the concentration of the extract was low at higher depths in the gel column than at the gel-liquid interface, it did not allow crystals to dissolve completely after the fragmentation, but retained a steady state, i.e., a balance between the growth and dissolution. The average length of crystals after fragmentation was found even less than 1 mm. After fragmentation, the dimensions of crystals remained far less than 5 mm, i.e., the maximum dimension of calculi which can pass through the urinary tract.

### 6.6.9 Total Mass and Volume of the Grown Struvite Crystals

After the growth and dissolution studies, the struvite crystals were gently removed from the gel and the total mass as well as the total volume of the crystals for each concentration was measured.
Figure 6.16 shows the histograms depicting the total mass and total volume of the grown crystals for each concentration of the extracts.

Both total mass and volume of the grown struvite crystals are considerably lower for the extracts in comparison to the control solution depicting the inhibitory effect of the extracts. The least mass and volume are observed in the case of 1.0 % C. wightii.

6.6.10 Statistical Analysis

The single factor analysis of variance (ANOVA) was carried out using MS excel to check the comparison of values of apparent length of struvite crystal in the control and each extract groups. ANOVA statistical analysis confirmed that the variations in the average length of struvite crystals with concentration as well as with time for each evaluated herbal extracts were highly significant at 0.05 level. From this in vitro growth inhibition study, it can be concluded that all the investigated herbal extracts i.e. B. diffusa, C. wightii and R. aquatica are found to be a potent inhibitor for struvite crystals.
6.7 Growth Inhibition Study by Fruit Juice of Citrus Medica Linn

A treatment with alkali, usually in the form of magnesium potassium citrate or potassium citrate, is very common to increase urinary citrate and reduce the rates of stone formations in the patients of hypocitraturic calcium nephrolithiasis [153-155]. A critical review on preventive treatment of nephrolithiasis with alkali citrate is written by Mattle and Hess [156]. Inasmuch as the most of the earlier studies on citrate inhibition have been mainly concentrated on calcium oxalate monohydrate and brushite crystals, the present investigation has been carried out to prove the citrate inhibition in struvite crystals also. For human being Acetohydroxamic acid (AHA) is the most widely used irreversible inhibitor of bacterial urease. AHA has a high renal clearance, can penetrate the bacterial cell wall, and acts synergistically with several antibiotics. Although in vivo studies have demonstrated that AHA inhibition of bacterial urease decreases urinary alkalinity and ammonia levels even in the presence of infection, 20 % of patients experience associated adverse effects. These include phlebitis, deep venous thrombosis, and hemolytic anemia. In addition, the use of AHA in patients with impaired renal function (serum creatinine level > 2.5 mg/dL) limits its effectiveness and increases its toxicity [157].

Struvite type kidney stones thrive in basic conditions of urine and hence the treatment should be the acidification of the urine. It is of prime importance to carry out the search for suitable struvite inhibitor, which has probably no side effects. As one of the main chemical constituents in the juice of Citrus medica Linn is citric acid, it has been decided to check its inhibitive effect on struvite crystals. Therefore, the growth inhibition study of struvite
crystals was carried out by the present researcher using natural fruit juice of *Citrus medica* Linn under *in vitro* conditions to identify the potency of its inhibition, which can be further, studied *in vivo*.

### 6.7.1 Single Diffusion Gel Growth Technique

For this study single diffusion gel growth technique was used and up to the process of gelation the same steps were followed as described in the sub section 6.6.1 of this chapter. After gelation took place, 20 mL supernatant solutions (SS) of pure 1.0 M magnesium acetate—\(\{\text{C}_4\text{H}_6\text{MgO}_4.4\text{H}_2\text{O}\}\) prepared with different concentration of fresh and filtered juice of *Citrus medica* Linn were gently poured on the set gels in test tubes. After pouring SS, the test tubes were capped with airtight stopples. Here, for each test tube, 20 mL SS of 1.0 M magnesium acetate were prepared by taking different volumes of the juice of *Citrus medica* Linn and distilled water. Composition and the pH of the SS are as shown in table 6.6.

**Table : 6.6 : Composition and pH of SS with Fruit Juice of *Citrus Medica* Linn**

<table>
<thead>
<tr>
<th>Number of Supernatant Solution (SS)</th>
<th>Composition of the Supernatant Solution (SS)</th>
<th>pH of the Supernatant Solution</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Volume (mL)</td>
<td>Powder (g)</td>
</tr>
<tr>
<td></td>
<td>Juice of <em>Citrus Medica</em> Linn</td>
<td>Distilled Water</td>
</tr>
<tr>
<td>SS-1</td>
<td>00</td>
<td>20</td>
</tr>
<tr>
<td>SS-2</td>
<td>02</td>
<td>18</td>
</tr>
<tr>
<td>SS-3</td>
<td>04</td>
<td>16</td>
</tr>
<tr>
<td>SS-4</td>
<td>06</td>
<td>14</td>
</tr>
<tr>
<td>SS-5</td>
<td>08</td>
<td>12</td>
</tr>
<tr>
<td>SS-6</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>SS-7</td>
<td>12</td>
<td>08</td>
</tr>
<tr>
<td>SS-8</td>
<td>14</td>
<td>06</td>
</tr>
<tr>
<td>SS-9</td>
<td>16</td>
<td>04</td>
</tr>
<tr>
<td>SS-10</td>
<td>18</td>
<td>02</td>
</tr>
<tr>
<td>SS-11</td>
<td>20</td>
<td>00</td>
</tr>
</tbody>
</table>
6.7.2 Struvite Crystals Grown in the Gel Media

Figure 6.17 shows the photographs of the struvite crystals grown in the gel medium. It is observed that as the concentration of the juice of *Citrus medica* Linn is increased in the SS, the number of struvite crystals grown in the silica hydro gel medium decreases and also average size of the struvite crystals decreases. This clearly indicates inhibition due to *Citrus medica* Linn.

![Figure 6.17 Photographs of the Struvite Crystals Grown in Gel Medium in Test Tubes with Different Concentration of the Juice of *Citrus Medica* Linn](image)

6.7.3 Growth of Struvite Crystals at Gel – Liquid Interface

After pouring of the SS, dendritic type crystals were grown in the gel at the gel–liquid interface. The growth rates of struvite crystals, at the end of 2\(^{nd}\) and 4\(^{th}\) day, growing in the gel at the gel–liquid interface for different concentration of SS are presented in table 6.7.

<table>
<thead>
<tr>
<th>Number of Supernatant Solution</th>
<th>Growth Rate (cm / day)</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>At the end of Day 2</td>
<td>At the end of Day 4</td>
<td></td>
</tr>
<tr>
<td>SS-1</td>
<td>0.608</td>
<td>0.309</td>
<td></td>
</tr>
<tr>
<td>SS-2</td>
<td>0.300</td>
<td>0.192</td>
<td></td>
</tr>
<tr>
<td>SS-3</td>
<td>0.250</td>
<td>0.125</td>
<td></td>
</tr>
<tr>
<td>SS-4</td>
<td>0.240</td>
<td>Dissolution Started</td>
<td></td>
</tr>
<tr>
<td>SS-5</td>
<td>0.225</td>
<td>Dissolution Started</td>
<td></td>
</tr>
<tr>
<td>SS-6</td>
<td>0.240</td>
<td>Dissolution Started</td>
<td></td>
</tr>
</tbody>
</table>
It can be noticed from table 6.7 that the growth rate of crystals and hence the size of the crystals are decreased with the increasing concentration of *Citrus medica* Linn. Here, the lower values of growth rate in comparison to control solution, the reductions in growth rates with increasing concentrations of the juice as well as the reduction in growth rates with time evidently proved the inhibitory effectiveness of the juice.

It was observed that the length of crystals growing in the gel at gel–liquid interface increased up to first 4 days in the cases of the SS-1 (i.e., control solution) and SS-2; and then they started dissolving. The length was increased up to first 3 days in the case of SS-3 and the dimension remained unchanged up to the end of 4th day; and then started dissolving. In the cases of the SS-4 to SS-6, length of the crystals growing in gel at gel–liquid interface increased just up to first 2 days; and then they started dissolving gradually.

It was remarkably found that in the case of SS-7 and for other higher concentrations, i.e., for SS-7 to SS-11, struvite crystals could not either nucleate or grow at the gel–liquid interface; which can be clearly noticed in the photographs in figure 6.17. This might be due to the effect of higher concentration of the juice of *Citrus medica* Linn in the SS. Thus, this study significantly proved the inhibitory potency of the *Citrus medica* Linn juice.

Figure 6.18 shows the histograms depicting the maximum apparent length of the grown struvite crystals in the gel media for different concentrations of SS. It is observed that the maximum dimensions of the grown crystals in the gel media decreases with the increasing concentration of *Citrus medica* Linn in the SS, which may be due to inhibitory effect of the juice.
6.7.4 Growth and Dissolution of Struvite Crystals at Gel–Liquid Interface

This study is important as it is conducted under the growth conditions. The simple dissolution is tested by placing the already grown crystal in an appropriate solution. The usual aim is to achieve the inhibition and dissolution of growing calculi in a body, where the required nutrients for the growth are being continuously supplied. Altogether, the same thing is mimicked in this *in vitro* experiment. At the gel–liquid interface the concentration gradients of the nutrients are the maximum and hence it is important to study the effect of juice on the growing crystals. The growth and dissolution of struvite at the gel–liquid interface is shown by the plots of average length *versus* time period in figure 6.19.

![Figure 6.18 Maximum Apparent Length of Dendritic Type Struvite Crystals](image)

**Figure : 6.18 Maximum Apparent Length of Dendritic Type Struvite Crystals**

![Figure 6.19 Growth and Dissolution of Struvite at the Gel–Liquid Interface](image)

**Figure : 6.19 Growth and Dissolution of Struvite at the Gel–Liquid Interface**
It is found that the crystals grown at the gel-liquid interface are dissolved up to a certain extent in control solution SS-1, which may be due to the formation of acetic acid. But, at the same time, it is observed that the dissolution of the crystals grown at the gel–liquid interface takes place comparatively faster for the higher concentrations of *Citrus medica* Linn, i.e., for the SS-2 to SS-6.

The dissolution rates of grown crystals in the gel at gel–liquid interface for the different concentration of *Citrus medica* Linn are as given in table 6.8.

**Table : 6.8 : Dissolution Rates of the Struvite Crystals in the Gel at Gel–Liquid Interface for the Different Concentrations**

<table>
<thead>
<tr>
<th>Dissolution Rates (cm / day)</th>
<th>No Inhibitor SS-1</th>
<th>Concentration of SS with Juice of <em>Citrus medica</em> Linn</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>SS-2</td>
</tr>
<tr>
<td>Dissolution Rate (cm / day)</td>
<td></td>
<td>1.79x10^{-2}</td>
</tr>
<tr>
<td>Enhanced Dissolution Rate</td>
<td></td>
<td>-</td>
</tr>
<tr>
<td>% of Enhanced Dissolution Rate</td>
<td></td>
<td>-</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Number of Days Required for Complete Dissolution of the Dendritic Type Struvite Crystals Grown at the Gel – Liquid Interface</th>
</tr>
</thead>
<tbody>
<tr>
<td>Days</td>
</tr>
</tbody>
</table>

Dissolution rates for SS-3 to SS-6 are found remarkably higher than that of control solution. Moreover, the dissolution rates are increased with the increasing concentration of juice in the SS, except the case of SS-2. Moreover, table 6.8 also represents enhanced dissolution rates as well as the percentage of enhanced dissolution rates due to the presence of juice. This further suggests that at the gel – liquid interface the already observed dissolution due to the formation of acetic acid is enhanced by the presence of juice. It is found that the percentage of enhanced dissolution rates increased appallingly with the increasing concentration of juice.
As mentioned in table 6.8, the number of days required for the complete dissolution of the dendritic type struvite crystals grown at the gel – liquid interface are reduced with the increasing concentration of juice. It is further noticed that minimum 8 days were required for the complete dissolution of crystals grown at gel – liquid interface for the concentration of SS-6.

### 6.7.5 Growth and Dissolution of Struvite Crystals at Different Depth

At different depths from the gel–liquid interface the grown crystals were measured and the phenomenon of the onset of dissolution was noted down. The growth and dissolution of struvite crystals grown at different depths in each cases from SS-2 to SS-11 are as shown in the plots of average length versus time in figures 6.20 (a-j). As the depth of the gel column increases, the average lengths of the grown crystals are decreased gradually by taking more time. It may be due to the lower concentration gradients at higher depths. Moreover, the average size of grown struvite crystals decreases with the increasing concentration of the juice in the SS. The period of dissolution, i.e., the time taken for the complete dissolution of the crystals, is found moderately less for the crystals grown near the interface, whereas it is quite longer for the crystals grown at the higher depth. At the gel–liquid interface the dissolution is faster due to the presence of sufficient amount of the juice. However, the amount of the juice decreases down towards the bottom of the gel column, which may be responsible for the delayed dissolution. It can be noticed from the figure 6.20 (j) for SS-11 that the dissolution period is the same for two different depths and average length of crystals are almost nearly same, which indicates that the higher amount of juice brings almost enough concentrations in the gel column to dissolve crystals nearly in uniform manner.
Figure: 6.20 (a) to (j) Growth and dissolution of Struvite at different depth from the gel–liquid interface
6.7.6 Depth of Dissolution

The depth of dissolution is defined as the depth from the gel – liquid interface up to which either the grown crystals are dissolved completely or no crystal can grow at all. Figure 6.21 shows the plots of depth of dissolution versus time in days. It is found that the depth of dissolution increases with time. In pure control solution SS-1, it is almost parallel to the x-axis and for solutions of higher concentrations of the juice it is pushed deeper and deeper into the gel. The depth of dissolution is increased slowly for the lower concentration, while it is increased rapidly for the higher concentrations, e.g., the depth of dissolution of 5.00 cm is achieved in just 15 days for SS-10 and SS-11; while it is achieved in 39 days for SS-4. Single factor ANOVA was also carried out; which established that the difference in the depth of dissolution was highly significant at 0.001 level.

6.7.7 Complete Dissolution

A histogram of figure 6.22 shows the numbers of days required for the complete dissolution of grown crystals in the gel media for different concentrations of SS. It is found that, the number of days required for the complete dissolution is reduced with the increasing concentration of the juice of Citrus medica Linn in the SS. For example, one can notice from the
histogram that it took 70 days for the complete dissolution for SS-2 and just 15 days for SS-11. Thus the reduction in the days for the complete dissolution with the increasing concentration of juice undoubtedly proved the potency of inhibition of struvite crystals.

6.8 Mechanism of Inhibition

Inhibition of crystal growth is a widely studied subject and hence a collection of research papers has been edited by Amjad [158] and published in a book form. Inhibition is a complex interplay where the crystal structure, nature and bonds exposed on plane considered, adsorption the crystalline face, chemical potentials and chemical reactivity, impeding the motion of growing steps on crystalline face, forming a complex of cations required for growth in solutions etc. have their important influence.

Considering brushite crystal as a good model, Skitric et al [159] summarized following points for interaction of additives with crystal.

1. The importance of molecular size and structure of the additive, i.e., small or macromolecules, number of functional groups in the molecule and its overall change in the growth of crystals. This may be useful in the selection of inhibiting molecule. For example, glutamate and aspartate ions have only one overall negative charge, which produce no significant effect on CHPD (brushite) crystal faces, which can be increased by attaching more negative groups like OH⁻.

2. The importance of structural fit between the organic molecule and the ionic structure of a particular crystal face. This decides the order of inhibition or reaction at particular crystalline face. This may differently affect various crystalline faces depending upon the crystalline face exposed to the solution
and as a result may change the morphology of the growing crystal. Small molecules with negatively charged groups, such as citrate ions do interact with the lateral faces of brushite crystals and slowing down the crystallization and inviting changes in the crystal morphology.

(3) The influence of the hydration layer exposed on the surface of the crystals. For polyaspartic acid and brushite crystals, such structural fit exists between distances of neighboring calcium ions from two adjacent layers constitute Ca-HPO$_4$ bi layer lying beneath hydrated layer parallel to the (0 1 0) plane.

Lundager Madsen and Bech Pederson [160] have found for brushite that most of the foreign metal ions are inhibitors, the only exception being Mg$^{2+}$, which has no significant effect and Pb$^{2+}$, which is a promoter. They have explained the inhibition and promotion mechanism as follows:

(1) Inhibition of crystal growth is due to adsorption of the inhibitor and so it is the inhibition of nucleation. The inhibitor is adsorbed to minute nuclei and preventing their further development.

(2) Promotion involves most likely the nucleation stage only, this stage being facilitated by the possibility of forming less soluble compound than that formed in the absence of additive. (For example, the promotive effect of Pb$^{2+}$ on both crystallization and hydrolysis of brushite may be due to nucleation of less soluble hydroxypyromorphite \{Pb$_5$OH(PO$_4$)$_3$\}.)

(3) Reduction of average crystal size in comparison with the blank may be caused by promotion of nucleation, inhibition of crystal growth, or both. Growth inhibition is usually accompanied by irregular growth, because adsorption is not uniform, or growth rate fluctuates in space and time. If the
degree of adsorption is different for different faces of crystal then habit modification occurs.

Wierzbicki et al [42] studied inhibition of struvite by phosphocitrate. The presence of phosphocitrate induced crystal face specific inhibition of struvite crystals, which lead to the total cessation of crystal growth when sufficient inhibitor concentration was available. The crystal growth studies and results of molecular modeling suggested strong affinity of phosphocitrate to (1 0 1) faces of struvite. As a consequence of this an alteration in the expression of these faces was observed which led to the characteristic narrowhead struvite morphology. However, the similar changes were not observed in the presence of identical concentrations of citrate, acetohydroxamic acid, and N-sulfo-2-amino tricarballylate, indicating the unique interaction of phosphocitrate with the struvite crystal lattice.

To assess the inhibiting behaviour of phosphocitrate on struvite Wierzbicki et al [42] used Cerius molecular modeling software. The initial confirmation was positioned near the struvite surface within electrostatic interaction range and the energy minimization procedure was applied. The minimization procedure considers the contributions from electrostatic faces, van der Waal forces, hydrogen bonds, bond angles and dihedral angles to the total energy of a system. However, the application of model was complicated due hemimorphic nature of struvite [161], for which the crystal habit does not reflect the full symmetry group of the crystal. In hemimorphic character of struvite, (0 0 1) faces are accompanied by larger (0 0 1̅) faces. Also (0 1 1) faces accompanied by smaller (0 1 1̅) faces. The crystal morphology is
completed by a set of \((1\ 0\ 1)\) faces accompanied by much smaller symmetry related \((1\ 0\ \bar{1})\) faces.

There are also growth inhibition studies reported for hydroxyapatite by aspartic acid \([162]\), heme crystals by antimalarials \([163]\), COM crystals from human kidney tissue culture medium \([164]\), calcium pyrophosphate crystals \([165]\). There are several models and mechanisms discussed by many authors, for instance, a model on complete crystal growth inhibition based on thermodynamics of interfaces \([166]\) and computation by numerical solutions to modified fully transient 2D continuum model of crystal growth for arrest by an adsorption – inhibition mechanism \([167]\).

The citrate inhibition theory has been widely discussed theory for urinary calculi inhibition. Citrate prevents crystallization by binding with calcium as well as citric acid brings the pH of solution to 3.0 to 4.0. Citrate is the most important complexor of calcium in urine and reduces ionic calcium concentration \([15, 168-170]\).

As mentioned earlier in section 2.7.3 of chapter II, hypocitraturia, a low amount of citrate in urine, is considered to be an important risk factor for urinary stone formation. Generally urinary citrate is considered as important inhibitor of the crystallization of stone forming calcium salts. The mean normal urinary citrate excretion is 640 mg / day in healthy individuals.

In the pathophysiology, the excretion of citrate in urine is a function of filtration, re-absorption, peritubular transport, and synthesis by the renal tubular cell. The proximal tubule reabsorbs most (70-90%) of the filtered citrate and as a result the citrate secretion is negligible. Altogether, acid-base status plays the most significant role in citrate excretion. Alkalosis enhances
citrate excretion, whereas acidosis decreases it. In acidosis, increased citrate utilization by the mitochondria in the tricarboxylic acid cycle occurs. This results in lower intracellular levels of citrate, facilitating citrato-re-absorption and hence reducing citrate excretion. Citrate excretion is generally impaired by acidosis, hypokalemia, a high–animal protein diet, and urinary tract infection [171].

Citrate plays several important roles in the mechanism of urinary calculi formation. First of all citrate binds with calcium ions in the urine and reduces calcium ion activity, consequently, the lowering the urinary supersaturation of calcium phosphate and calcium oxalate. Second, citrate has a direct inhibitory effect on the crystallization and precipitation of calcium salts. In addition, citrate may also enhance the effectiveness of protein inhibitors of crystallization. For example, inhibition of aggregation of COM crystals by Tamm-Horsfall protein (THP) is increased by citrate. Citrate also expected to reduce the urinary osteopontin, which is an important component of the protein matrix of urinary calculi [172]. Moreover, urinary citrate excretion also increases urinary pH, which is a factor in uric acid crystallization and uric acid stone formation [171].

De Yoreo et al [173] carried out the combination of two investigative tools, namely, AFM and molecular modeling and discussed both the physical and stereochemical factors responsible for COM inhibition by citrate. Molecular modeling investigations of interactions of citrate with steps and faces on COM crystal surfaces provided links between the stereochemistry of interaction and the binding energy levels that underlie mechanisms of growth modification and changes in overall crystal morphology.
The citrate inhibition suggests that sodium citrate, citric acid and other citrate compounds are acting as alkalization agents indicated for systematic metabolic acidosis (renal tubular acidosis), while urinary alkalization or hypocitraturia contains di-sodium citrate. Pak et al [168] reported successful management of uric acid nephrolithiasis with potassium citrate. Potassium citrate reduces urinary saturation of calcium salts by complexing calcium and reducing ionic calcium concentration. Later on Pak [174] preferred potassium citrate because it appeared to decrease urinary calcium excretion at least transiently. Similarly, sodium citrate [175], potassium citrate [176], sodium potassium citrate [177], and potassium-magnesium citrate [178] were studied. In present author’s lab, earlier workers studied in vitro growth inhibition of brushite crystals by citric acid [49,179,180].

Many juices have been tried to study the risk factor of urolithiasis. The effect of cranberry juice on urinary risk factor for calcium oxalate stones has been investigated by McHary et al [181], which exhibited antilithogenic properties. Effect of acute load of grapefruit juice on urinary excretion of citrate and urinary risk factors for renal stone formation has been discussed by Trinchieri et al [182]. Moreover, effect of blackcurrant, cranberry and plum juice consumption on risk factors associated with kidney stone has been reported [183]. As cranberry juice acidifies urine it could be useful in the treatment of brushite and struvite stones. Blackcurrant juice can support the treatment and metaphylaxis of uric acid stone disease because of its alkalizing effect. Lemon juice [180] and lemonade [184] have been found useful in management of urolithiasis. A comparative study of orange juice versus lemonade in reducing stone forming risk is reported [185].
In the present investigation of growth inhibition of struvite crystals by selected herbal extracts, the present author proposes two stage inhibition hypothesis. These herbal extracts contain several forms of sterol, lipids, organic acids etc. and they are expected to inhibit the growing struvite crystals in two different stages:

(1) By forming stable complexes with Mg\(^{2+}\) ion and thus impeding the supply of Mg\(^{2+}\) ions for the growth of struvite crystals. (For instance, Magnesium-L-Aspartate Dihydrate \(\text{C}_8\text{H}_{12}\text{O}_8\text{N}_2\text{Mg}.2\text{H}_2\text{O}\) is the magnesium salt of aspartic acid and like other magnesium salts of organic acids, is highly water soluble.)

(2) By adsorption on the growing crystalline surface. The large amount of adsorption of organic compound may induce desorption of complex formed by Mg\(^{2+}\) ions and thereby weakening the bonds of crystals leading to fragmentation.

These herbal extracts contain different types of organic compounds and it is difficult to pin point directly which compound is responsible for growth inhibition of struvite crystals.

It is proposed by the present author that the citric acid present in the Citrus medica Linn is responsible for growth inhibition of struvite crystals. The other acids and sterols may be responsible for forming complexes of Mg\(^{2+}\) and thereby not allowing it for growth of struvite crystals. The higher concentration of juice contains higher concentration of citric acid which may dissolve struvite by forming a soluble magnesium citrate. Thus the present study may also support citrate inhibition theory for struvite crystals, which has been already proved for calcium oxalate and brushite crystals.
6.9 Conclusions

1. The comparative lower values of growth rate, reductions in the growth rates with the increasing concentrations of extracts as well as the reduction in the growth rates with the time proved the inhibitory effect of all the three evaluated extracts, i.e. *Boerhaavia diffusa* Linn, *Commiphora wightii* and *Rotula aquatica* Lour.

2. The maximum apparent lengths of the dendritic type struvite crystals grown at the gel – liquid interface were reduced with the increasing concentration of each tested extract.

3. Significant percentage of inhibition of growing struvite crystals at gel – liquid interface at the end of first day was noticed in each of the herbal extracts, clearly demonstrating the inhibitory effect of all the three extracts used for the investigation. The maximum percentage of inhibition 46.16 % was observed for 1.0 % *R. aquatica* extract, whereas minimum 13.33 % of inhibition was observed for 0.5 % of *B. diffusa* extract.

4. Dissolution rates for all the cases with the herbal extracts were found remarkably higher than that of control solution. Moreover, dissolution rates significantly increased with the increasing concentration of herbal extract, except for *R. aquatica*. Enhanced dissolution rates were observed for all the herbal extracts. Maximum percentage of enhanced dissolution rate 138.8 % was observed in the case of 1.0 % *B. diffusa* Linn.

5. The dendritic type struvite crystals grown at the gel – liquid interface were found to be dissolved completely within 21 to 40 days in all the cases with herbal extract. Minimum 21 days were required for the complete dissolution for 1.0 % *B. diffusa*. 
6. From the analysis of growth and dissolution rates, it was found that extracts of *R. aquatica* have retarded the growth rate from the very beginning of the crystal growth, whereas extracts of *B. diffusa* have speed up the dissolution rates once the growth of the crystals took place.

7. All the extracts impeded the diffusion process of reactants occurring in the gel column for the nucleation and, subsequently, the growth of crystals. The reduction in depths of growth indicated the inhibition offered by all the three herbal extracts.

8. Growth rates as well as the apparent size of the crystals grown at the higher depths were lower for all the cases with herbal extracts.

9. The average dimensions of the prismatic type crystals grown with the herbal extracts were comparatively smaller than that of control solution, which also gave an idea of the inhibitory effect of all the three evaluated extracts. The least average dimension of prismatic crystals was found to be 0.1 cm for the 1.0 % *C. wightii* extract.

10. The remarkable phenomenon of fragmentation of the grown struvite crystals was observed for all the three tested herbal extracts. The average length of crystals after fragmentation was found even less than 1 mm.

11. Both the total mass and volume of the grown struvite crystals were considerably lower for the cases with extracts depicting the inhibitory effect of all the evaluated herbal extracts. The least mass and volume observed for 1.0 % *C. wightii*.

12. As the concentration of the juice of *Citrus medica* Linn was increased in the SS, the number of struvite crystals grown in the gel medium decreased and also average size of the struvite crystals decreased.
13. Lower values of growth rate in comparison to control case, reductions in the growth rates with the increasing concentrations of the juice as well as the reduction in growth rates with the time evidently proved the inhibitory effectiveness of the juice.

14. Struvite crystals could not either nucleate or grow at the gel–liquid interface for the higher concentration of juice in the SS (e.g. SS-7 to SS-11), which significantly proved the inhibitory potency of the *Citrus medica* Linn juice.

15. Maximum dimensions of the grown crystals in the gel media decreased with the increasing concentration of the juice in the SS, which might be due to inhibitory effect of the *Citrus medica* Linn juice.

16. Dissolution rates of the crystals grown at gel-liquid interface for SS-3 to SS-6 were found remarkably higher than that of control solution. Moreover, the dissolution rates were increased with the increasing concentration of the juice in the SS, except the case of SS-2.

17. Enhanced dissolution rates were observed for all the concentration with the *Citrus medica* Linn juice.

18. The period of dissolution of the crystals was found moderately less for the crystals grown near the gel–liquid interface, whereas it was quite longer for the crystals grown at the higher depth from the gel–liquid interface.

19. The depth of dissolution was increased slowly for the lower concentration, while it was increased rapidly for the higher concentration of the juice.

Single factor ANOVA statistical analysis established that the difference in the depth of dissolution was highly significant at 0.001 level.
All the struvite crystals grown in the gel were found to be dissolved completely within 15 to 55 days in all the cases with the juice. Minimum 15 days were required for the complete dissolution in the cases of SS-10 and SS-11. Moreover, reduction in the number of days required for the complete dissolution with the increasing concentration of the juice undoubtedly proved the potency of inhibition of struvite crystals.

From this in vitro growth inhibition study of struvite crystals, it can be concluded that all the investigated herbal extracts i.e. *B. diffusa*, *C. wightii* and *R. aquatica* as well the juice of *Citrus medica* Linn are found to be potent inhibitors for struvite crystals.
CHAPTER VI: Growth Inhibition Study of Struvite

References


145. Web site: www.ayushveda.com


