Discussion
**Discussion**

Kidney stone is a common chronic disorder affecting 10-15% of the general population worldwide. Calcium-containing stones are the most common comprising about 75% of all urinary calculi, which may be in the form of pure calcium oxalate (50%) or calcium phosphate (5%) and a mixture of both (45%). Calcium oxalate stones are found in two different varieties, calcium oxalate monohydrate (COM) or Whewellite, and calcium oxalate dihydrate (COD) or Weddellite. COM, the thermodynamically most stable form, is observed more frequently in clinical stones than COD and it has greater affinity for renal tubular cells, thus responsible for the formation of stones in kidney [163].

Many factors affect the growth of urinary calculi. Different mineral metabolisms are important in the formation of urinary stones or calculi [164]. The urinary calculi are composed of mainly crystalline components. Multiple steps are involved in the formation of the crystals, which are nucleation, growth and aggregation. The saturation state of body fluids with respect to stone-forming constituents and the presence of various biomolecules (inhibitors/stimulators) in the body fluids as well as organic matrix are known to influence mineralization [165, 166, 167, 168]. The matrix displays a variable and complex composition and a few proteins of matrix are common in various stones. It is observed that certain macromolecules isolated from normal urine (i.e., healthy individuals) inhibit COM crystal growth *in vitro*. In normal individuals, kidney stone formation is suppressed by these urinary inhibitors [169] and some of such inhibitors are proteins.

Extracorporeal shock wave lithotripsy (ESWL) is currently the first-line treatment for upper urinary tract calculi. This treatment is not without side effects [170] and kidney damage during ESWL is a clinically significant problem [171]. The mechanisms underlying shockwave-induced renal tubular injury are not completely understood, though shear forces, thermal and cavitation effects, and free radical formation have been postulated [172, 173]. Therefore, it is worthwhile to look for an alternative for the management of urolithiasis.
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Today, about 80% of the world population residing in third world countries still relies almost entirely on plant products for their primary health care. The remaining 20% of individuals living in the first world use, in more than 25% of cases, pharmaceuticals which have been directly derived from plant products [174]. Many medicinal plants have been employed during ages to treat urinary stones though the rationale behind their use is not well established through systematic and pharmacological studies, except for some composite herbal drugs and plants [175, 137]. Interestingly, the areas having high consumption of these plant products, reported a very low incidence of urolithiasis and dietary patterns have been thought to play an important role for varied incidence of urinary calculi in the specific regions [176].

Seeds of *Trachyspermum ammi* (L.) Sprague ex Turril (Umbelliferae) locally named as Ajwain in India, is commonly used in folklore to treat urolithiasis. So far, its diuretic properties have been documented in literature and it is actively used in various drug formulations of kidney stone treatments [15, 16]. Till date, various plant extracts have been studied to reduce the incidence of calcium stone deposition both in vivo and in vitro [177, 178, 179] but the identification of naturally occurring CaOx inhibitory biomolecules from plants was hampered in past by limitation in identification method. In the present study, in vitro and in vivo efficacy of *Trachyspermum ammi* antilithiatic protein (TAP) on calcium oxalate and calcium phosphate crystallization was evaluated.

4.1. *In vitro efficacy of Trachyspermum ammi antilithiatic protein (TAP)*

When the homogeneous system of *in vitro* mineralization was employed under physiological conditions of temperature, pH and ionic strength of the media, the Ca$^{2+}$ and HPO$_4^{2-}$ ions got precipitated as solid mineral phase in the formation of hydroxyapatite [Ca$_{10}$(PO$_4$)$_6$(OH)$_2$] [168]. Using such a system, the effect of all the three plants on the initial mineral phase formation, its subsequent growth and demineralization of the preformed mineral phase was investigated. Usually the plant extracts possessing antilithiatic properties exert their action on the body by altering ionic composition of urine [180]. Possibly for *in vitro* system, the plant extracts alter ionic concentration of Ca$^{2+}$ and HPO$_4^{2-}$ may be by stereospecifically regulating mineralization of calcium.
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containing crystals, as most aspartic acid rich proteins like uropontin do in vitro [118] thereby decrease their precipitation.

The inhibitory human proteins preventing further growth of calcium stones in kidneys do so by binding to the surface of calcium crystals and thus preventing further aggregation of calcium salt precipitates [181]. We can hypothetically predict that plants extract under experimentation contains such biomolecule(s) which not only prevent initial nucleation of calcium phosphate precipitation but also have such biomolecule(s) which bind to or get absorbed at crystal phase surface of already formed crystals and thereby block the growth sites. Both Trachyspermum ammi and Rubia cordifolia have quite similar ability to inhibit initial mineral phase (Figure 3.1), however, Rubia cordifolia shows very low inhibitory ability to inhibit the growth of the preformed mineral phase as compared to initial mineralization (Figure 3.2). This might be due to absence of such crystal binding biomolecule(s) in its plant extract.

Demineralization is a process of releasing Ca$^{2+}$ and HPO$_4^{2-}$ ions from its bound precipitated form to free state. Here, again Trachyspermum ammi showed maximum dissolution of preformed calcium phosphate into the aqueous phase (Figure 3.3).

Trachyspermum ammi showed maximum inhibitory potential towards initial mineral phase formation, its subsequent growth and demineralization of the preformed mineral phase as compared to other two plants. Correspondingly, the seeds of Trachyspermum ammi were further tested for their activity towards CaOx crystal growth. The aqueous extract of Trachyspermum ammi was found to be highly effective in inhibiting CaOx crystal growth in vitro and its ability to inhibit the growth was proportional to the volume of extract used, higher volume showed better inhibition.

The in vitro study was further preceded towards the purification of the most effective antilithiatic biomolecule from the seeds of Trachyspermum ammi. The qualitative identification of biomolecules having antilithiatic property in the seeds of Trachyspermum ammi was done after fractionation of aqueous extract of Trachyspermum
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*Ammi* into fractions having more than and less than 10 kDa molecular weight biomolecules (more than 10 kDa fraction and less than 10 kDa fraction).

The fraction having more than 10 kDa molecular weight biomolecules was found to have higher inhibitory potency as compared to the less than 10 kDa molecular weight biomolecules extract. In addition, it was found that the more than 10 kDa extract had higher inhibitory potency as compared to its crude aqueous extract at identical concentration. A significant increase in its ability to inhibit initial mineral phase formation after the fractionation of crude extract suggest that in the fresh sample the active biomolecule(s) may be present bound to other molecules and during dialysis they are released in the free form which is more active and stable.

The more than 10 kDa fraction of *Trachyspermum ammi* was further tested for the presence of various phytochemicals and it showed presence of proteins in it. Thus, a protein purification strategy was adopted to further proceed for the purification of the most potent antilithiatic protein.

**4.2. Identification and characterization of antilithiatic protein from the seeds of *Trachyspermum ammi***

There are various stone inhibitory proteins [182, 183, 118] which are present in urine, having similar physical and chemical properties. Most of these proteins have been isolated from CaOx kidney stones matrix itself in their active form [165, 166, 167]. Likewise, many plants are also known to produce CaOx as crystalline deposits [89, 90], having an organic matrix constituting different proteins [184]. These proteins are believed to play an important role in the control of crystal growth and modification of crystal form [185]. More recently [120] four proteins from the organic matrix of CaOx crystals present in the seeds of *Phaseolus vulgaris*, have been isolated which inhibited the nucleation of CaOx crystallization in solutions. So, it is worthwhile to look for a CaOx inhibitory protein from the seeds of *Trachyspermum ammi* since it showed strong antilithiatic properties.
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In the present study an antilithiatic protein was isolated from the seeds of *Trachyspermum ammi* inhibiting both calcium oxalate and calcium phosphate crystallization. The purification was performed systematically, including ammonium sulfate precipitation, anionic adsorption and finally size separation. Among several fractions that had inhibitory activity against CaOx crystal growth, the fraction which eluted at 980-1072 minutes as shown in figure 3.9, showed maximum percentage inhibition. Characterization and functional analyses of other fractions will have additional significant impact on new modulators of stone formation.

The purified *Trachyspermum ammi* antilithiatic protein (TAP) has a molecular mass of 107 kDa and pI of 6.2. The purified TAP had an absorbance maximum at 280 nm and it lacked significant light absorption in visible region. The peptide mass fingerprinting analysis of the isolated protein showed maximum similarity (44% sequence coverage) with an unnamed protein of *Vitis vinifera* (UPVV) (CAO23876). Although molecular weight of UPVV (87 kDa) is not similar with TAP protein, but the pI of TAP is comparable with UPVV. Since, many plant databases are still largely incomplete, many proteins present in *Trachyspermum ammi* are absent in those databases. So, TAP is not homologous with UPVV, but probably is UPVV like protein.

On exploring the inhibitory potency of TAP on calcium oxalate and calcium phosphate crystallization, an effective inhibition was observed, thus clearly indicating that TAP is probably imparting its effect by binding to calcium ions. This emphasizes the prospect that TAP has a calcium binding site, which is responsible for its ability to inhibit calcium oxalate and calcium phosphate crystallization.

Many higher plants have shown to accumulate crystalline calcium oxalate, which have an organic matrix constituting different proteins including CaOx inhibitory proteins [120]. *Vitis vinifera* have also shown presence of such needle-shaped CaOx crystals in them [109] indicating the possible existence of CaOx inhibitory proteins in it. The genome of *Vitis vinifera* has been recently sequenced [186], so it has many proteins whose putative function and name is yet to be assigned. On doing BLASTp of unnamed protein product of *Vitis vinifera*, its similarity was identified with calcium binding EF
hand proteins (Figure 3.19). Two EF hand domains were also identified in it by SMART (Figure 3.20). This clearly indicated that TAP is also a putative calcium binding protein having EF hand domains in it.

The calcium binding proteins that have been characterized by high-resolution X-ray crystal structure analysis fall into two general categories [187]. One group includes many extracellular enzymes and proteins that have enhanced thermal stability or resistance to proteolytic degradation as a result of binding calcium ions. The other group is made up of a family of intracellular proteins that reversibly bind calcium ions. The second group is distinguished from the first in that its members have common calcium binding helix-loop-helix motif, termed an "EF-hand" that has been widely applied to describe calcium binding sites [188]. It has been suggested by Mustafi and Nakagawa [189] that most of kidney stone inhibitory proteins like nephrocalcin are similar to the proteins of the second group because they reversibly binds calcium ions. In addition few known CaOx inhibitory proteins like osteonectin and calgranulin [190] have also showed presence of calcium binding EF hand domains in them [191,192].

The two EF hand domains identified were further subjected to in silico studies to understand the mechanism of action involved in interaction of COM with these domains. In silico studies were accomplished by docking of both EF hand domains with COM unit cell. After docking simulations, it was found that both EF hand domains interact strongly (negative docking score) with COM crystal. In addition it was also observed that acidic amino acids in the EF hand domains were mainly involved in providing this strong interaction because the oxygen atom of the carboxyl group forms a strong bond with calcium atom of COM crystal (shown by LIGPLOT analysis). It is a fact that hydrogen-bonding (or ionic bonding) are of primary significance in establishing strong complex between ligand and protein active binding site, nevertheless hydrophobic interactions also act as a stabilizing factor and addition of a hydrophobic group not only allows hydrophobic bonding but also strengthens existing bonds and the increased bond strength can be an important factor in determining the overall binding energy [193].
Both EF hand domains have the ability to strongly interact with free available growing sites (Ca, C and O atom) (Figure 2.5) of COM. It is known that COM crystal growth is slow in some directions since certain macromolecules adsorb on it and prevent formation of crystal lattice. Face (-101) of COM crystals is more active as it presents more closely packed calcium atoms and has significantly more adsorptive characteristics for many macromolecules (proteins) [194]. In the present investigation, it was observed that calcium ions of COM form bonds with mainly acidic amino acids and carbon of oxalate group gets involved in hydrophobic interactions with mainly aliphatic amino acids.

The interaction of amino acids is also dependent on the conformation of the active sites, same amino acid in one instance is effectively involved in hydrogen bonding and in other instance same amino acid is involved in week hydrophobic interactions as is shown in the case of EF hand domain a. Furthermore, EF hand a have two consecutive Glu at postition 713 and 714 but the Glu at 714 is only involved in forming both hydrogen bonding and hydrophobic interactions. This dependence is purely steric hindrance, thus suggesting that not all amino acids which could strongly bind with calcium ions, although repeatedly present in the active binding site, interact with COM. Thus there is no advantage of repeated Glu residues until they are structurally available to interact. Strong hydrogen bonding of Glu with calcium ion of COM crystal supports the hypothesis that acidic amino acids which are negatively charged are attracted to positively charged calcium ions [195].

However, as stated by Wesson et al. [196], the charge of the side group is not the sole determinant to cause this effect since not all Glu present in this binding site interacted with COM. This evidence further suggests that conformational and interface chemistries interact in a complex manner to inhibit aggregation of COM and an understanding of such interactions may help to determine and control the factors affecting kidney stone formation.

The role of acidic amino acids like aspartic acid and glutamic acid on CaOx inhibition is known since long time [197]. It has also been suggested that acidic amino
acid residues such as Asp and Glu, that are expected to be deprotonated and negatively charged at urinary pH, are attracted to positively charged calcium ions of calcium stones [198]. Our data of amino acid analysis suggests that TAP has higher acidic amino acids (Asp and Glu) content. Thus, it could be argued that TAP, which is a UPVV like protein possess the capability to reduce calcium crystallization.

A recent study by Wang [199] presented that addition of serine spacer in poly aspartate peptide increased their ability to restrain COM crystallization. They suggested that the hydroxyl groups (-OH) of serine may have contributed in the interaction by directly binding to calcium ions and formation of ionic bonds. A significant amount of serine amino acids in TAP further ascertain its ability to impede CaOx crystallization.

A plant protein from the seeds of *Trachyspermum ammi* was shown to attain the ability of inhibiting CaOx crystallization *in vitro*. The protein was anionic in nature having abundant acidic amino acids and a similarity of this protein with an unnamed protein of *Vitis vinifera* was also found. Due to this similarity, presence of two EF hand domains in TAP was anticipated, signifying its calcium binding properties which is a feature of most kidney stone inhibitory proteins. Since, all these findings were accomplished with *in vitro* study which is a static system; the observed effect would be obviously many folds more during dynamic *in vivo* system where there is a continuous draining of water by efficient urinary system of our body. Thus, evaluation of *Trachyspermum ammi* antilithiatic protein (TAP) on rat hyperoxaluric model was conducted to investigate its antilithiatic potential *in vivo*.

4.3. *In vivo* characterization of TAP using rat hyperoxaluric model

The hyperoxaluric model used in the present investigation was designed by Yamaguchi et al [158]. They proposed different experimental conditions to study various phases of calcium oxalate stone formation in rats. To evaluate the formation of many stone formation in the kidney within little time ethylene glycol (0.4%) is administered with ammonium chloride (1.0%). They found that after 9 days of this particular dose, urine of these rats was saturated with both COD and COM stones and the kidney tissue
showed little injury whereas after 15 days of the same dose, the number of the urine remained saturated with both COM and COD crystals and the kidney tissue showed marked injury. Thus, to evaluate the efficacy of the isolated protein (TAP) on CaOx crystallization in vitro, 9 days treatment was given and to evaluate the ability of TAP to reduce oxalate caused renal injury, 15 days treatment was given.

A decrease in the body weight of rats given ethylene glycol (EG) and ammonium chloride was observed after 9 days treatment and the 15 days treatment showed a higher degree of this body weight reduction. It is known that EG in vivo gets metabolized to various toxic metabolites. It is also suggested that EG consumption leads to multiorgan injuries and concentrations above 0.75% may produce metabolic acidosis [200, 201, 202]. The decrease in the body weight of these rats could have been due to the effect of other metabolites of EG on the whole body. Thus, the decrease in the body weight cannot be related to kidney stone formation. In contrast, the treatment of these hyperoxaluric rats with TAP showed normalization of body weight, which implies that TAP might have an ability to interact with those toxic metabolites of EG which could have caused the deleterious effect on the body weight.

There was no significant difference in the urine volume of hyperoxaluric rats exposed to EG + NH₄Cl and the hyperoxaluric rats administered TAP. The increase in urine volume after EG dose was a protective mechanism adopted by the kidneys of rats to expel out excess of CaOx crystals from their body. Trachyspermum ammi did not interfere with this protective mechanism of body and thus the urine volume remained high. In addition, the rats given TAP at dose 2 mg per kg body weight for 15 days showed a significant increase in the urinary volume as compared to hyperoxaluric rats. This rise in urinary volume could be attributed to the diuretic properties of TAP [15, 16].

The renal tubular enzymes in 24 hour urine are treated as sensitive index of renal tubular damage. So, we studied the effect of TAP on EG-induced changes in the excretion of two renal tubular enzymes i.e. alkaline phosphatase (AP) and lactate dehydrogenase (LDH). AP and LDH are two cytosolic enzymes and their higher activity in the extracellular fluid indicates cell lysis. The enhanced urinary excretion of renal
injury marker enzymes like AP and LDH in urolithiatic animals suggests damage to the brush border membrane of the renal tubules. This damage also appears to associate with the retention and deposition of crystals in the kidneys [203]. Recent, in vitro studies have suggested that proximal tubule cells, when compared to distal tubule or collecting duct cells, are more sensitive to the toxic effects of both oxalate and calcium oxalate at pathological level [204]. Wiessner also showed that coating crystals with urinary macromolecules enhanced the attachment of the crystals to injured renal cells at a pH of less than 6.0 [205]. Studies show that crystal formation results in cell damage and cell detachment from the basement membrane, and the released degradation products can promote heterogeneous nucleation of calcium salts such as calcium oxalate and calcium phosphate.

The tissue injury which occurred upon administration of EG and NH₄Cl resulted in increase of COM deposition in kidney tissue (Figure 3.33). The animals given EG and NH₄Cl dose for 15 days showed much higher excretion of renal injury markers enzymes than 9 days treatment (Figure 3.26 & 3.27). This is because EG results in many other toxic metabolites in addition to oxalic acid and exposure to these toxins for longer duration would results in higher order of renal injury [203]. TAP restored renal injury in the EG and NH₄Cl exposed animals after treatment of both 9 and 15 days. This shows that in addition to reducing COM crystal and oxalate in rat kidneys, TAP has some additional properties of reducing toxic effects of other metabolites of ethylene glycol.

Further evidence for efficacy of TAP towards restoration of hyperoxaluric manifestations comes from urinary crystal analysis. It is known that free and aggregated calcium oxalate monohydrate crystals are being excreted in hyperoxaluric and recurrent stone formers, respectively [206], whereas single crystals of calcium oxalate dihydrate and few calcium phosphate are excreted in normal subjects [207,208]. Among these, calcium oxalate monohydrate crystals have greater affinity for renal tubular cell and are responsible for the formation of stone [209]. The formation of calcium oxalate dihydrate and calcium phosphate in preference to calcium oxalate monohydrate crystals is propitious because it protects against stone disease by reducing the attachment of crystals.
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to renal tubular cells. The above observation was found unchanged after 9 days treatment of hyperoxaluric rats with TAP, where the urine excreted in 24 hours was found to be predominant with COD crystals. This might be due to the ability of the protein (TAP) to prevent the crystallization of calcium oxalate to COM or due to ability to TAP to convert conformation of COM to COD. In addition, the size of COD crystals in the hyperoxaluric animals given a higher dose of TAP was markedly small, thus depicting their ability to get easily excreted. After 15 days of treatment period the urine of rats treated with TAP presented disintegrated COD crystals (Figure 3.29d). This further ascertained the ability of TAP in having a direct/indirect effect on calcium oxalate crystals to either reduce its growth or disintegrate its structure.

Increased serum urea is an important manifestation of kidney stone disease. Renal dysfunction further diminishes the ability to filter urea and increases serum urea level [210]. Although the EG treated rats showed an increased urinary volume as a prophylactic adjustment to filter toxic metabolites from the blood, but still the level of urea in serum was found high in EG and NH$_4$Cl exposed rats after both 9 and 15 days treatment. Depending on the magnitude of renal dysfunction, the level of serum urea was more after 15 days treatment than after 9 days treatment. After EG and NH$_4$Cl exposure the serum urea level increased, indicating renal dysfunction due to crystal deposition. Here, again rebalancing of serum urea further unveils the potential effect of TAP on maintaining renal functioning.

Creatinine clearance (CrCl) is a clinically accepted index to measure glomerular filtration rate (GFR). Any alteration in GFR indicates the state of kidney functioning. Creatinine clearance measures the volume of blood plasma that is cleared of creatinine per unit time. A marked decrease in CrCl after EG + NH$_4$Cl exposure indicates disability of kidneys to filter out creatinine, thus depicting renal dysfunction. It has also been found that external prophylactic agents restore renal functioning by maintaining creatinine clearance and serum urea level in hyperoxaluric rats [211]. Similarly, in our study TAP restored renal functioning upon its administration. The impairment of renal functioning in EG + NH$_4$Cl exposed rats could be attributed to observed renal injury in them and
improvement in renal functioning in these rats after TAP administration also confirmed decrease in renal injury.

Crystal retention within the renal tubules is promoted by renal epithelial injury, which exposes a variety of crystal adhesion molecules on epithelial surfaces, including CD44 and its ligands OPN and hyaluronic acid [212, 213, 214]. OPN was observed to be upregulated in EG-induced CaOx nephrolithiasis showing its highest expression in tubules [203]. Similarly in our case, most of the crystal deposition occurred in the renal tubules (Figure 3.35). Bowman capsule and glomerulus showed no crystal deposition, but distortion was found in their structure (Figure 3.35b). Oxalate is readily filterable at glomerulus and secreted by proximal tubules [215, 216]. The damage of glomerulus and its capsule following oxalate exposure might have been caused by oxalate itself or its derivative(s) by acting as free radicals.

The crystals are first formed in the renal proximal tubules, as calcium in calcium oxalate crystals is derived from the glomerular filtrate [217]. In humans, various changes in urine chemistry, including hyperoxaluria, hypercalciuria and hypocitraturia, can lead to the development of abundant crystals within the renal tubules. Using calculations based on the concentration of ions in the renal tubules, Finlayson and Reid [218] reported that crystals are not usually retained and could not reach a size large enough to occlude the tubular lumen within the urinary transit time. In normal kidneys, it takes 3 min for urine to pass from the glomerulus to the renal pelvis; it would take several hours for crystals to become large enough to obstruct a collecting duct [218], suggesting that unless calcium oxalate crystals bind to the tubular membrane surface, stone development would not be possible. In agreement with Finlayson and Reid, the present studies showed that hyperoxaluria-induced renal tubular damage is associated with crystal attachment and subsequent aggregation and growth of calcium oxalate kidney stones.