Summary & Conclusion
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Urolithiasis is a problem that has challenged clinicians since the time of Hippocrates, and many family physicians have extensive experience in its clinical management. In the recent years, technological advancements have greatly facilitated the diagnosis of stone disease. Among six main types of commonly occurring stones, the calcium stones are most abundant comprising about 75% of all urinary calculi. Calcium oxalate calculi is one of the major clinical problems estimated to afflict approximately 12% of the population, with a high recurrence rate of 70–80% in males and 47–60% in females [221]. 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. Many factors affect the growth of urinary calculi. 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].

Present day medical management of urolithiasis mainly involves techniques like extracorporeal shock wave lithotripsy (ESWL) and percutaneous nephrolithotomy (PCNL); however, the prevention of recurrence of stone formation is not assured. Besides, these treatments cause undesirable side effects such as hemorrhage, hypertension, tubular necrosis and subsequent fibrosis of the kidney leading to cell injury and recurrence of renal stone formation [222]. Cell injury provokes the retention of calcium oxalate crystals, which forms the nidus and grows by a cascade of events leading to stone formation. Because of the morbidity and mortality of these surgical procedures, some oral drugs are used to treat this disease but adverse effects compromise their long-term consumption. On the other hand due to the adverse effects of these drugs, alternative treatment modalities composed of herbal remedies have been the mainstay of medical therapy for thousands of years, especially in Eastern civilizations. Although it is believed that the resurgence of interest in phytotherapy became popular in the second half of the 19th
century in Western countries, this complementary medical therapy was widely used in Europe much before that date.

Natural product compounds are the source of numerous therapeutic agents. Progress to discover drugs from natural product sources has resulted in compounds that are being developed to treat resistant bacteria and viruses, cancer and immunosuppressive disorders. Recent years have shown a dramatic expansion in the knowledge of molecular mechanism of phytotherapeutic agents used to treat urolithiasis. The discovery and elucidation of the mechanism of action, in particular the clinical role of these herbal remedies, has made an important contribution to the treatment for urinary stone disease as an alternative or adjunct therapy. Although phytotherapeutic extracts are popular in folk culture, because of the absence of scientific data on the exact clinical role, efficacy and side effects of these herbs the potential consumption of this alternative medical therapy as an alternative or adjunct to classic therapy remains to be determined. In this respect, scientific research designed to determine the exact mechanism of action of these drugs would be fruitful.

In the present research, in vitro and in vivo properties of Trachyspermum ammi have been evaluated. 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 [15, 16] and it is actively used in various drug formulations of kidney stone treatments. 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. Initially, in vitro properties of Trachyspermum ammi were compared with Rubia cordifolia and Zingiber officinale. Further, from the seeds of Trachyspermum ammi an antilithic protein was isolated and characterized. Finally, the efficacy of the purified protein was evaluated using in vivo hyperoxaluric rat model. The conclusions made from results obtained at every step of the study are summarized point wise.
1. First of all the efficacy of *Trachyspermum ammi* was compared with other two antilithiatic plants *i.e.* *Rubia cordifolia* and *Zingiber officinale*. In vitro comparative studies were conducted on the above three plants towards initiation of CaP mineral phase, growth of CaP over its preformed mineral phase and finally demineralization of CaP preformed mineral phase. Among all the three plants, *Trachyspermum ammi* showed the maximum ability to inhibit both initiation and growth of CaP mineralization, additionally, it exhibited highest potential towards demineralization of preformed mineral phase. Further, the crude extract of *Trachyspermum ammi* was fractionated into more than and less than 10 kDa biomolecule fractions. Qualitative examination of biomolecules present in the more than 10 kDa fraction (having maximum inhibitory potency) revealed presence of proteins.

2. Since, more than 10 kDa protein fraction of *Trachyspermum ammi*, was found to possess significant antilithiatic properties, therefore, a three step purification procedure was adopted to purify it. The sequential three step purification procedure starts with ammonium sulfate, anion exchange and molecular sieve chromatography. At each step, the activity of fractions was tested for CaP and CaOx inhibitory potency and finally the most potent antilithiatic fraction was purified in its pure form. The purity of this fraction was also confirmed using RP-HPLC. It was found that as the fraction having highest inhibitory potential towards CaP and CaOx was purified, its activity increased at each consecutive step.

3. Further, the *Trachyspermum ammi* antilithiatic protein (TAP) was characterized. The molecular weight of this protein as determined by size exclusion HPLC was found to be 107 kDa. The *Trachyspermum ammi* antilithiatic protein has an isoelectric point of 6.9 and $\lambda_{\text{max}}$ at 280 nm. The total amino acid analysis of TAP revealed relatively high presence of acidic amino acids like Glu (12.18%) and Asp (9.4%). Mascot search engine analysis of m/z ratios obtained from trypsinized peptide mass fingerprinting of TAP presented the similarity of TAP
with an unnamed protein product of *Vitis vinifera* (CAO23876). The sequence coverage of TAP with this protein was found to be 44%.

4. Extending the characterization, putative function and active domains of this unnamed protein product of *Vitis vinifera* (UPVV) were identified. By SMART normal module, two EF hand domains and one LETM1 domain were identified in it. EF hand domain is a helix-loop-helix motif which is a known calcium binding domain. Additionally, certain known kidney stone inhibitory proteins like calgranulin and osteonectin have also shown the presence of such EF hand domains in them. So, the CaOx and CaP inhibitory activity of TAP is assumed to be imparted by such EF hand domains in it (due to its significant similarity with UPVV). In silico studies were done to compare the affinity of both EF hand domains and elucidate their mechanism of binding with COM crystal. It was found that both EF hand domains have a negative free energy of binding, indicating its strong interaction with COM crystal. In addition it was also found that acidic amino acid Glu was responsible for forming strong bond with the calcium atom of COM crystal. The role of acidic amino acids like aspartic acid and glutamic acid on CaOx inhibition is acknowledged long back [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].

5. Finally, the efficacy of TAP was studied on rat hyperoxaluric model. TAP restored the level of creatinine clearance, urinary injury marker enzymes and content of serum urca & creatinine in hyperoxaluric rats. The effect of TAP on urinary crystallization revealed that TAP in a dose-dependant manner resulted in decrease of urinary crystallization. Moreover, TAP administration resulted in a marked decrease in crystallization of kidney tissue and reduction in kidney tissue histological alterations. This effect of TAP was dose-dependant as a higher dose showed better restoration.
Summary and conclusion

In summary, *Trachyspermum ammi* has an ability to inhibit stone formation under both *in vitro* and *in vivo* conditions. A protein from the seeds of *Trachyspermum ammi* has been shown to possess the ability to inhibit calcium phosphate and calcium oxalate crystallization. This protein which is anionic in nature has abundant acidic amino acids and further its similarity with an unnamed protein of *Vitis vinifera* is found. Due to this similarity, presence of two EF hand domains in TAP is anticipated, signifying its calcium binding properties which is a feature of most kidney stone inhibitory proteins. Activity of this protein from *Trachyspermum ammi* adds a new dimension to kidney stone treatment.