MATERIAL & METHOD
3.1. Collection of human renal stone sample and its extraction

Human renal calculi, surgically removed from the kidney stone patients were obtained from the Department of Urology, Postgraduate Institute of Medical Education and Research (PGIMER), Chandigarh, India. Stones were taken from those patients who were more than 25 years of age and were suffering from no other abnormality, and the stones were of a non-infectious nature. After FTIR analysis, the stones with calcium and oxalate as their major components were selected for further studies.

3.1.1. Protein extraction

3.1.1.1. EGTA extraction

Proteins were isolated from the matrix of kidney stones containing CaOx as the major constituent using EGTA as a demineralizing agent. Stones were washed in 0.15M NaCl with gentle stirring for 48 hours to remove the adhered blood, tissue etc. They were then dried and pulverized with a mortar and pestle. For extraction of the organic matrix of powdered stone, each gram of stone was suspended in 10mL of 0.05M EGTA, 1mM PMSF and 1% β-mercaptoethanol. The extraction was carried out for 4 days at 4°C with constant stirring. The suspension was centrifuged for 30 minutes at 10,000g and at 4°C. The supernatant of EGTA extract was filtered through Amicon ultra centrifugal filter device with a molecular weight cut off 10,000 daltons at 4°C and concentrated to a known volume. Whole EGTA extract, greater than and less than 10,000 daltons fractions were stored at -20°C for further studies (Aggarwal 2000).

3.1.1.2. SDS extraction

For SDS method, 1g stone powder was mixed with 4mL SDS reducing buffer (0.06M Tris HCl, pH6.8, 10% glycerol, 5% β-mercaptoethanol, and 2% SDS) and heated
(100°C, 30min). Samples were centrifuged at 700g for 15 minutes at 4°C and the supernatant collected. The supernatant was filtered through Amicon ultra centrifugal filter device with a molecular weight cut off 3,000 daltons at 4°C to remove SDS and concentrated to a known volume (Williams et al. 2006).

3.1.1.3. Acetic acid extraction

For the acetic acid method, 1gm stone powder was vortexed with 5 mL 10% acetic acid and incubated for 30 min. Samples were centrifuged at 3,000g for 20 min at 4°C and the supernatant was retained and filtered as described above (Williams et al. 2006).

3.1.2. Lipid extraction

Lipids were extracted from stone powder by the method of S R Khan et al. (Khan et al. 2002). 1 gm of stone was extracted in 30 mL of ice-cold chloroform: methanol: 0.05 mol/L Tris-HCl, pH 7.4 (2:1:1); using sonication at 4°C. After sonication the sample was centrifuged at 10,000g to phase separate and pellet residue. The upper and lower phases were removed separately and pooled as aqueous and organic substances, respectively. Extraction solvent was added to the pellet and the process was repeated several times pooling the respective phases. Pooled organic phases were extracted with ethanol: ether (3:1) and then centrifuged. Non-complexed lipids were isolated from the supernatant. Complexed lipids were recovered from the pellet by dissolving it into DMSO by sonication. Complexed lipid in DMSO was preserved at -20°C for further use.

3.1.3. Separation of biomolecules on the basis of their molecular weight
Renal stone extract obtained through EGTA extraction was centrifuged by using Amicon Ultra-4 centrifugal separating tubes (Millipore) of 10 kDa cut off molecular weight. Thus, two fractions >10kDa and <10 kDa were obtained. Whole EGTA extract, >10 kDa and <10kDa fraction were assayed on different assay system described below.

3.1.4. Protein concentration

Total protein concentration of the supernatant of renal stone matrix obtained by EGTA extraction method as mentioned above was determined using a commercial Bradford assay reagent (Bio-Rad Laboratories, Hercules CA).

Principle

The assay (Bradford 1976) is based on the observation that the absorbance maximum for an acidic solution of Coomassie Brilliant Blue G-250 shifts from 465 nm to 595 nm when binding to protein occurs. Both hydrophobic and ionic interactions stabilize the anionic form of the dye, causing a visible color change. The assay is useful since the extinction coefficient of a dye-albumin complex solution is constant over a 10-fold concentration range.

Reagents

1. Bradford reagent: 100 mg Coomassie Brilliant Blue G-250 was dissolved in 50 mL of 95% ethanol. To this solution 100 mL of 85% (w/v) phosphoric acid was added. Resultant solution was diluted to 1 liter and filtered through Whatman #1 paper just before use.

2. 1 M NaOH

Procedure
• 0.1mL of test solution was taken in a test tube.

• Equal volume of 1 M NaOH was added to each sample and was mixed.

• Finally, 5 mL of Bradford reagent was added and the solution was mixed and incubated at room temperature for 5 minutes.

• Similarly standard and blank tubes were prepared and absorbance of all samples was measured at 595 nm.

**Calculation**

The concentration of protein (μg/mL) was calculated using following formula

\[
\frac{\text{Absorbance of test}}{\text{Absorbance of standard}} \times \text{concentration of standard}
\]

**3.1.5. Homogeneous assay system of initial mineral phase of calcium phosphate**

Homogenous mineralization system was used to study the extent of *in vitro* mineral phase formation to determine the activity of calcium phosphate (CaP) precipitation (Kabra et al. 1978). The homogenous system consisted of 5mM CaCl₂ and 5mM KH₂PO₄. After incubating this system at 37°C, precipitates obtained were centrifuged and the pellets were resuspended in 0.1N HCl. The calcium (Ca²⁺) and phosphate ions (HPO₄²⁻) concentration in the precipitate represented the extent of precipitation (crystallization) of these ions and the inhibitory biomolecule(s) will minimize the extent of their precipitation. The Ca²⁺ and HPO₄²⁻ ions were estimated by the methods of Trinder (Trinder 1960) and Gomori (Gomori 1941) respectively. Percentage inhibition or stimulation of mineral phase in the presence of renal stone extract (whole EGTA extract, >10kDa & <10kDa fraction) was calculated as:
%age Inhibition = \left[ \frac{(C-T)}{C} \right] \times 100$, where $T$ is the concentration of Ca$^{2+}$ or HPO$_4^{2-}$ ion of the precipitate formed in the assay system with the renal stone extract or lipid extract and $C$ is the concentration of Ca$^{2+}$ or HPO$_4^{2-}$ ion of the precipitate formed in control system which had distilled water (Millipore).

3.1.5.1. Determination of calcium

Method of Trinder was used to determine the calcium ions in a sample.

Principle

Calcium ions get precipitated as naphthyl hydroxamate by directly adding slight excess of calcium reagent. After centrifugation excess of reagent is removed by decantation and unwashed precipitates are dissolved in EDTA. Addition of ferric nitrate results in development of an orange red color, intensity of which is measured as the amount of calcium ions present in the sample.

Reagents

- Calcium reagent- This reagent was prepared by mixing two components and the volume was raised to 1000mL by adding distilled water, the resultant mixture was filtered and stored in dark reagent bottle. The two components were as follows:

  (a) 280 mg of Naphthylhydroxamic acid in 100 mL of (95mL distilled water + 5mL ethanolamine + 2 gm of tartaric acid).

  (b) 9 gm of NaCl in 500 mL of distilled water.
- Color reagent- 60 gms FeNO₃ was dissolved in 500 mL of acidified distilled water (485 mL distil water with 15 mL of conc. HNO₃). Then, the volume was raised to 1000 mL with distilled water.

- EDTA- 2 gm EDTA was dissolved in 1000 mL of 0.1 N NaOH.

- Working standard- 2mM of calcium chloride.

**Procedure**

- 0.1mL of sample was dissolved in 2.5 mL of calcium reagent and incubated at room temperature for 30 minutes.

- The precipitate were obtained after centrifugation and then dissolved in 1.0 mL of 0.2% EDTA with boiling for 10 minutes.

- Finally, 3 mL coloring reagent was added and the absorbance was measured at 450 nm.

**Calculations**

The following formula was used to calculate the concentration of calcium ions (mM).

\[
\frac{\text{Absorbance of test}}{\text{Absorbance of standard}} \times \text{concentration of standard}
\]

**3.1.5.2. Determination of phosphate**
The phosphate ions concentration in the sample was determined by the method of Gomori.

**Principle**

Phosphate reacts with molybdic acid to form phosphomolybdic acid. Treatment of 2-methyl-4 aminosulfate causes reduction of phosphomolybdic acid to form deep blue colored complex which gives absorption maxima at 660 nm.

**Reagents**

- Molybdic acid- Prepared by mixing 2.5% ammonium molybdate dissolved in distilled water and 10 N H$_2$SO$_4$ in the ratio of 10:4.

- Metol reagent- Prepared by mixing 5% NaHSO$_3$ and 1% metol in distilled water.

- Working standard- 2mM of KH$_2$PO$_4$

**Procedure**

- 1.2 mL of molybdic acid was added to 0.2 mL of sample and incubated at room temperature for 10 minutes. The sample was diluted by 6.8 mL of distilled water.

- Then, 0.5 mL of metol reagent was added and the solutions were mixed properly.

- Finally the mixture was kept at room temperature for 20-30 minutes and absorbance was measured at 660 nm.

**Calculations**

The following formula was used to calculate the concentration of phosphate ions (mM).
3.1.5.3. Determination of percentage inhibition of calcium phosphate mineral phase

The percentage inhibition of CaP mineralization was calculated using following formula.

\[
\text{\% age inhibition} = \left[ \frac{(C-T)}{C} \right] \times 100
\]

where, `C` is the concentration of \( \text{Ca}^{2+} \) or \( \text{HPO}_4^{2-} \) ions of the precipitate formed in control system which had distilled water. `T` is the concentration of \( \text{Ca}^{2+} \) or \( \text{HPO}_4^{2-} \) ions of the precipitate formed in assay system with the test sample.

3.1.6. Homogeneous assay system of growth and demineralization of calcium phosphate mineral phase

The growth and demineralization of preformed mineral phase consisting of calcium phosphate required initial precipitates of these minerals as obtained by the initiation of calcium phosphate mineral phase. To study the growth of the preformed mineral phase, the precipitates formed by the above method were resuspended in the same assay system having calcium and phosphate along with the three fractions of kidney stone extract. This assay system was incubated at 37°C for 30 minutes. Then \( \text{Ca}^{2+} \) and \( \text{HPO}_4^{2-} \) ions were estimated and the concentration of these ions represented the growth of precipitation of these ions over the previously formed mineral phase.

For demineralization, the preformed mineral phase was resuspended in the assay system with all the three fractions of kidney stone extract but without further addition of calcium and phosphate ions. This assay system was incubated at 37°C for 30 minutes. \( \text{Ca}^{2+} \)
and HPO$_4^{2-}$ ions were estimated in supernatant to determine the demineralization of mineral phase by all the three fractions of kidney stone extract.

Different volumes of renal extract were added in the homogenous assay system compensated with water. Five test tubes were taken for every volume of stone extract. The concentration of calcium and phosphate ions of mineral phase in these test samples was determined. In case of growth of pre-formed mineral phase, concentration of Ca$^{2+}$ and HPO$_4^{2-}$ ions was deducted from the final concentration of Ca$^{2+}$ and HPO$_4^{2-}$ ions. The percentage inhibition or stimulation caused by different fractions of renal extract was calculated with respect to control system which had distilled water instead of kidney stone extract. In case of demineralization, the percentage inhibition of Ca$^{2+}$ and HPO$_4^{2-}$ ions demineralized, was calculated in supernatant.

3.1.7. Calcium oxalate crystal growth

Inhibitory activity against CaOx crystal growth was measured using the seeded, solution depletion assay (Chutipogtanate et al. 2005). COM crystal seed (from FTIR identified clinical kidney stones) was added to a solution containing 1mM calcium chloride (CaCl$_2$) and 1mM sodium oxalate (Na$_2$C$_2$O$_4$). The reaction of CaCl$_2$ and Na$_2$C$_2$O$_4$ with crystal seed would lead to deposition of CaOx on the crystal surfaces, thereby decreasing free oxalate that is detectable at $\lambda$ 214 nm. When a protein is added into this solution, depletion of free oxalate ions will decrease if the protein inhibits CaOx crystal growth. Rate of reduction of free oxalate was calculated using the baseline value and the value after 30 second incubation with or without protein. The relative inhibitory activity was calculated as follows:
% Relative inhibitory activity = \[\frac{(C-S)}{C}\times 100\]

where, \(C\) is the rate of reduction of free oxalate without any protein and \(S\) is the rate of reduction of free oxalate with a test protein.

### 3.2. Purification of potent protein

#### 3.2.1. Materials

Materials required were Macro Prep 25 Q strong anion exchanger (Bio-Rad laboratories), Bio gel P-100 gel (Medium), and column (50x1 cm).

#### 3.2.2. Extraction

Only calci um oxalate renal stones confirmed after FTIR analysis were taken for the study. Protein extract was obtained through EGTA extraction method as described in section 3.1.1.1.

56.46 gms of calcium oxalate stones were collected. These stones were washed with 0.15 N NaCl. After drying at room temperature renal stones were powdered with the help of pestle and mortar. Renal powder was then extracted with EGTA extraction buffer (0.05M EGTA, 1mM PMSF and 1% β-mercaptoethanol). The extract thus obtained was centrifuged at 8000 rpm for 20 minutes at 4°C. Supernatant was separated into >10 kDa and <10 kDa fractions by centrifugation with the help of Amicon Ultra-4 centrifugal separating tubes (Millipore) of 10 kDa cut off molecular weight.

#### 3.2.3. Protein estimation

Protein concentration of whole EGTA extract, >10 kDa and <10 kDa fraction was estimated by Bradford assay (Bradford 1976).
3.2.4. Ion exchange chromatography

3.2.4.1. Sample

The fraction which exhibited highest activity against calcium oxalate and calcium phosphate assay system was filtered through Whatman paper. The sample obtained was lyophilized and was preserved at -80°C.

3.2.4.2. Preparation of column

- **Washing of slurry**

Macro Prep 25 Q strong anion exchanger support was supplied hydrated in 20% (v/v) ethanol. 80 mL of exchanger was taken into a beaker and was allowed to settle for 2-3 hrs. Ethanol solution was decanted. Matrix was washed with 2-3 bed volume of deionized water.

- **Equilibration of slurry**

After washing, matrix was mixed with starting buffer i.e. buffer A (20mM TrisCl +0.1M NaCl) having pH 7.4. After 2-3 wash with starting buffer pH of the slurry was checked with pH strips. When the color of pH strip matched with that of pH 7, equilibration of matrix was confirmed.

- **Column packing**

One third of column (50X1cm) was filled with equilibrating buffer. Supernatant of slurry was decanted and was mixed well. A funnel was kept at the top of column and slurry was poured over it slowly until the slurry reached the bottom of the column. After the column was half filled slurry was poured drop wise. For even packing bottom lid of the column was opened for the flow of packing buffer. When one third of the column was packed funnel was removed slowly and slurry was allowed to settle for 7-8 hours.
3.2.4.3. Sample loading & separation of biomolecules

Lyophilized sample was reconstituted with 2.75 mL of starting buffer and was loaded into the column. The bound protein was eluted with 0-90% of linear gradient of buffer A and B. Buffer A consists of 20mM Tris-Cl with 0.1M NaCl (pH 7.4) and Buffer B consists of 20mM Tris-Cl with 1.0 M NaCl (pH 7.4). Protein elution was done at flow rate of 1.0 mL/min using Automated Biologic LP system. Elution profile was made using LP data view software version 1.03. The absorbance of each fraction was read at 280 nm, simultaneously the conductance was also measured. The method used for anion exchange chromatography is illustrated in table 3.1

Fractions coming under the peak were pooled and dialyzed. Their activity was checked on calcium oxalate crystallization followed by SDS-PAGE run. Fraction which was showing highest activity and few bands was then lyophilized and was preserved for further purification.
Table 3.1 Method used for anion exchange chromatography.

<table>
<thead>
<tr>
<th>Time (minutes)</th>
<th>Flow rate (mL/min)</th>
<th>Buffer</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.0-25</td>
<td>1.0</td>
<td>Buffer A</td>
</tr>
<tr>
<td>25-65</td>
<td>1.0</td>
<td>0-90% gradient of buffer A to buffer B</td>
</tr>
<tr>
<td>65-85</td>
<td>1.0</td>
<td>Buffer B</td>
</tr>
<tr>
<td>85-155</td>
<td>1.0</td>
<td>Buffer A</td>
</tr>
</tbody>
</table>

3.2.5. Molecular sieve chromatography

3.2.5.1. Preparation of the gel

10 gram of Bio-Gel P-100 Gel (Medium) was added to 20mM Tris HCl buffer in a beaker. It was allowed to hydrate for 12 hours at 20°C. After hydration was completed, half
of the supernatant was decanted and the hydrated gel was degassed for 5-10 minutes with occasional swirling. Two bed volume of degassed buffer was added to degassed gel.

3.2.5.2. Column packing

Column packing was done by using the same method as described in section 3.2.4.2 column packing for ion exchange chromatography.

3.2.5.3. Sample loading & separation of biomolecules

Fraction showing highest activity was concentrated and loaded on a Bio gel® P-100 gel molecular sieve column (50X1cm) equilibrated and eluted with the 20 mM Tris buffer (pH 7.4) at a flow rate of 0.1mL/min. The fractions which eluted out based on their molecular weights were pooled to study their activity on calcium oxalate crystal growth assay system. Potent fractions thus obtained were subjected to SDS-PAGE and reverse phase HPLC.

3.2.6. Electrophoresis

For SDS-PAGE, lyophilized samples were reconstituted in reducing sample buffer and analyzed by one-dimensional discontinuous SDS-PAGE using 1 mm thick, 12% separating and 4.4% stacking gels with a Mini-Protean III apparatus (Bio-Rad Laboratories). Protein bands were stained with silver using ProteoSilver™ Plus Silver Stain Kit (PROTSIL2, Sigma-Aldrich Co.). Broad range molecular weight markers (catalog # 161-0317, Bio-Rad) were used as standards.
Table 3.2 Method used for molecular sieve chromatography

<table>
<thead>
<tr>
<th>Time (minutes)</th>
<th>Flow rate (mL/min)</th>
<th>Buffer</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.00-1984</td>
<td>0.10</td>
<td></td>
</tr>
</tbody>
</table>

3.2.7. RP-HPLC for homogeneity

Waters Spherisorb® C18 (5 μ, 4.6 X 250 mm) column with solvent A (0.1% TFA in water) and solvent B (100% acetonitrile containing 0.1% TFA) was used for determining the homogeneity of purified protein. Flow rate was maintained at 1 mL/min at the time of protein injection. The column was washed with solvent A and brought to 20% acetonitrile in 5 min. The bound protein was eluted with a linear gradient of acetonitrile (20-70 %) over a period of 50 min. The detection was monitored at 280 nm using Waters 2996 photodiode array detector.

3.2.8. Tryptic in gel-digestion of purified protein

Single band detected after molecular sieve chromatography was excised from the gel and was destained with destainer provided in the ProtcoSilver™ Plus Silver Stain Kit (PROTSL2, Sigma-Aldrich Co.). Trypsin profile IGD kit (PPO100, Sigma-Aldrich Co.) was used for in gel digestion of purified protein. Destained gel piece was dried for approximately 15 to 30 minutes. Trypsin solubilised in 1mM HCl and mixed with 40mM ammonium bicarbonate and 9% acetonitrile was added to the destained gel piece. Gel piece was fully covered by the addition of 40mM ammonium bicarbonate and 9% acetonitrile (pH 8.2) solution and was incubated for 5 hours at 37°C. After the incubation, liquid was removed from the gel piece
and transferred to a new labeled eppendorf tubes and was preserved for mass spectroscopic analysis.

3.2.9. Peptide mass fingerprinting by MALDI-TOF-MS

The proteolytic sample obtained after in gel digestion was premixed 1:2 with the matrix solution (α-Cyano-4-hydroxycinnamic acid) and spotted on the sample stage. It was dried at room temperature then washed with 0.1% TFA and was analyzed by Ultraflex TOF/TOF mass spectrometer (Bruker Daltonics, Germany (Bremen)). The mass/charge spectra obtained were searched in MASCOT search engine (http://www.matrixscience.com). All the three databases (MSDB, SwissProt, NCBI nr) available in the search engine was used. For search peptides were assumed monoisotopic, oxidized at methionine residues and carbamidomethylated at cysteine residues. Since we used human renal stones therefore, *Homo sapiens* taxonomy restriction was chosen, only one missed cleavage was allowed, and peptide mass tolerance of 1.2 kDa was used for peptide mass fingerprinting.

3.3. In silico study

3.3.1. Materials

Schrodinger software package was used for molecular modeling of identified protein. Calcium oxalate structure was drawn with the help of Molecular Operation Environment (MOE) software package (Chemical Computing Group, Montreal, Canada). After docking,
LIGPLOT program was used for describing the interactions between ligand and protein within a docked structure.

3.3.2. Homology modeling of identified protein

Homology modeling of the identified human phosphate cytidylyltransferase 1, choline, beta protein structure was modeled by using Prime module of Schrodinger (Prime version 1.5, Macromodel version 9.1, Schrodinger, LLC, New York, NY, 2005) software. The structure of protein was modeled on the basis of its structural similarity with the chain A CTP: glycerol-3 phosphate cytidylyltransferase of Bacillus subtilis (Protein Data Bank IC 1 COZ-A). The degree of identity between the template and the human CCT sequence was 31%, which enabled a preliminary model to be generated by Schrodinger. The sequence alignment was then improved manually and comparative homology method was used to build the structure.

3.3.3. Docking of Homology model of human CCT1 choline beta with calcium oxalate

Calcium oxalate structure was drawn with the help of molecular builder of MOE software developed by the chemical computing group inc. Montreal, Canada. Active site of the modeled protein was predicted by using active site finder tool of MOE software. Then docking of modeled protein with calcium oxalate was done in MOE-Dock. The docking energy calculation was carried out within a user-specified three-dimensional docking box (3D docking box) using the simulated annealing method under the MMFF94 X force field. The energy grids for docking were generated as grid-based potential fields by the MOE-Dock program, to reduce the calculation time.
Each docking energy value was calculated as the sum value of the electrostatic, Van der Waals, and flexibility energies. The interaction energy was calculated using the electrostatic and Van der Waals potential fields sampled on a grid overlaying the 3D docking box. The 3D docking box was interpolated at the atom positions by tri-linear interpolation. The Van der Waals parameters were taken from the currently active force field. The electrostatic field was calculated in the Coulombic manner using the current dielectric. MOE-Dock performed 25 independent docking runs, and wrote the resulting conformations and their energies to a molecular database file. The lowest docking energy conformation for each active site was chosen for LIGPLOT.

3.3.4. Point mutation of acidic amino acid of active site with neutral and positively charged amino acid

To further confirm the role of acidic amino acid in the inhibition process of kidney stone formation, they were point mutated with alanine, glycine, lysine, arginine and histidine in all the active site of the modeled protein. After mutation, protein was docked with calcium oxalate using MOE-Dock with same parameter of docking as was used for wild type.

3.3.5. Quality check of modeled protein

To check the quality of the modeled protein, Ramachandran Plot was drawn from the program Schrodinger, which checks the stereochemical quality of a protein structures, producing a number of postscript plots, analyzing its overall and residue-by-residue geometry, assured the reliability of the modeled protein.