CHAPTER 3
MATERIALS & METHODS

3.1. Collection of human renal stone sample and protein 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 Fourier transform infrared spectroscopy (FTIR) analysis, the stones with calcium and oxalate as their major components were selected for further studies.

3.1.1. Protein extraction from human Calcium Oxalate Stones: EGTA extraction

Proteins were isolated from the matrix of kidney stones containing calcium oxalate (CaOx) as the major constituent using Ethylene glycol-bis(2-aminoethylether)-N,N,N’,N’-tetraacetic acid (EGTA) as a demineralizing agent. Stones were washed in 0.15 M Sodium Chloride (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 10 mL of 0.05 M Ethylene Glycol Tetraacetic Acid (EGTA), 1 mM Phenylmethane Sulphonyl Flouride (PMSF) and 1% β-mercaptoethanol (βME). 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 and was stored at -20°C for further studies [Aggarwal et al., 2000].
3.1.2. Separation of biomolecules on the basis of their molecular weight

The protein extract obtained through EGTA extraction of human renal CaOx stones was filtered through Amicon ultra centrifugal filter device (Millipore) with a molecular weight cut off 3,000 daltons at 4°C and concentrated to a known volume. Whole EGTA extract and greater than 3 kDa fractions were stored at -20°C for further studies [Aggarwal et al., 2000].

3.1.3. Protein concentration (Lowry’s Method)

The Lowry’s method was used for the quantitative estimation of proteins [Lowry et al., 1951].

**Principle:** The phenolic group of tyrosine and tryptophan residues in a protein produces a blue-purple color complex with Folin-Ciocalteau reagent which has sodium tungstate molybdate and phosphate. Thus, the intensity of the color depends on the amount of aromatic amino acids present and the absorption is measured at 750 nm.

**Reagents:**

- Bovine Serum Albumin (BSA): 1 mg/mL stock solution
- Lowry A: 2% Sodium Carbonate (Na₂CO₃) in 0.1 N NaOH solution
- Lowry B: 1% Copper Sulphate (CuSO₄) in distilled water
- Lowry C: 2% Sodium Potassium Tartrate (KNaC₄H₄O₆·4H₂O) in distilled water
- Lowry’s Reagent: It was prepared by mixing 98 mL of Lowry A, 1 mL Lowry B and 1 mL Lowry C reagent
- Folin-Ciocalteau Reagent (1 N): Prepared fresh by diluting the commercial reagent with water in 1:1 ratio

**Procedure:**

- 0.9 mL water was added to 0.1 mL test sample.
- 5 mL Lowry’s reagent was added to it and vortexed followed by incubation at room temperature for 10 min.
• Finally, 0.5 mL Folin-Ciocalteau reagent was added. The solution was mixed again and incubated at 37°C for 30 min. Similarly, standard and blank tubes were run in parallel and then absorbance was taken at 750 nm.

Calculations:
Conc. of protein (mg/mL) = (Absorbance of test/Absorbance of standard) X Conc. Of Standard

3.1.4. Calcium oxalate (CaOx) crystals Nucleation
The method used was similar to that described by Hennequin et al. with some minor modifications [Hennequin et al., 1993]. Solutions of calcium chloride (CaCl₂) and sodium oxalate (Na₂C₂O₄) were prepared at the final concentration of 3 mmol/L and 0.5 mmol/L, respectively, in a buffer containing Tris Cl 0.05 mol/L and 0.15 mol/L NaCl at pH 6.5. Both solutions were filtered through a 0.22 μm filter (Millipore); 1.5 mL of CaCl₂ solution was mixed with different concentrations of extracted proteins. Crystallization was started by adding 1.5 mL of Na₂C₂O₄ solution. The final solution was stirred at 37°C repeatedly after an interval of 60 s for 8 min. The absorbance of the solution was monitored at 620 nm after every 60 s. The percentage inhibition produced by the protein extract was calculated as [1- (Tsi / Tsc)] × 100, where Tsc was the turbidity slope of the control and Tsi the turbidity slope in the presence of the inhibitor or promoter.

3.1.5. Calcium oxalate crystal growth
Inhibitory activity against CaOx crystal growth was measured using the seeded, solution depletion assay [Nakagawa et al., 1985]. Calcium oxalate crystal seed (from FTIR identified clinical kidney stones) was added to a solution containing 1 mM CaCl₂ and 1 mM Na₂C₂O₄. The reaction of CaCl₂ and Na₂C₂O₄ with crystal seed would lead to deposition of CaOx on the crystal surfaces, thereby decreasing free oxalate that is detectable at λ 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 s incubation with or without protein. The relative inhibitory activity was calculated as follows: \[ \text{Relative inhibitory activity} = \left( \frac{(C-S)}{C} \right) \times 100 \], 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.

### 3.1.6. Data Analysis

The data are represented as the mean ± standard deviation of three replicates (* p < 0.05).

### 3.2. Cell culture studies

#### 3.2.1. Cell culture

Normal dog epithelial derived renal tubular epithelial, Madin–Darby Canine Kidney (MDCK) cells were obtained from National Centre of Cell Sciences (NCCS, Pune). Chemicals such as Dulbecco’s modified Eagles’s medium (DMEM), fetal bovine serum (FBS), antibiotics, 0.25% Trypsin-EDTA, Sodium bicarbonate were used for the maintenance of the cell lines. The cells were maintained as monolayers in DMEM with 2.0 mM L-glutamine adjusted to contain 3.7 g/L sodium bicarbonate, 4.5 g/L glucose. Media was supplemented with 1% Penicillin (10,000 units/mL)-Streptomycin (10,000 µg/mL) and 10% FBS. Cells were cultured in 25 cm² tissue-culture treated flasks at 37°C and 5% CO₂ in humidified chambers [Aggarwal et al., 2010; Aggarwal et al., 2013b].

#### 3.2.2. Oxalate-induced cell injury

For experimental purposes, the MDCK cells were seeded into 96-well plates (1 × 10⁴ cells/well). Following plating a 24 h recovery for the cells to resume exponential growth at 37°C in a humidified incubator containing 5% CO₂, the cells were subjected to various treatments. For oxalate injury, cells were treated with 1 mM Sodium oxalate in DMEM (without FBS). The effect of the proteins in the presence of oxalate injury was assessed by adding proteins at various
concentrations in the presence of oxalate, to the cells. Cell injury was assessed by measuring the cell viability through various cell viability assays viz. lactate dehydrogenase (LDH) leakage into the medium, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) colorimetric assay, sulphorhodamine B (SRB) protein staining assay and Acidine Orange/Ethidium Bromide (AO/EB) Staining to Detect Apoptosis [Aggarwal et al., 2013c]

3.2.3. Preparation of the protein samples
For cell culture studies, the proteins was dialyzed through Millipore Amicon Ultra Centrifugal Filters (cut off 3 kDa) and desalted by ReadyPrep 2-D Cleanup Kit. The desalted protein sample was reconstituted in distilled water and was filtered using Millipore Millex GV Filter Unit 0.22 μm. This was treated as a stock solution of the proteins, stored at -20°C.

3.2.4. Lactate dehydrogenase (LDH) leakage assay
The cytotoxicity of the protein extract was assessed by LDH leakage (Butler, 2004). The MDCK cells were seeded into a 96-well plates (1 × 10^4 cells/well). Following plating and a 24 h recovery for the cells to resume exponential growth at 37°C in a humidified incubator containing 5% CO₂ the cells were subjected to various treatments. Cells of the control group were cultured in 100 μL of DMEM medium only (without FBS). For oxalate injury, cells were treated with 1 mM oxalate in DMEM. The effect of the proteins in the presence of oxalate injury was assessed by adding purified proteins at various concentrations in the presence of oxalate, to the cells. To verify whether the proteins by themselves contributed to cytotoxicity, cells were also incubated with the various concentrations of the proteins in the absence of oxalate. After 48 hours of treatment the LDH leakage was measured at 340 nm [Lee HG et al., 2010].

**Principle:** Lactate dehydrogenase (LDH) is an enzyme (E.C. 1.1.1.27) found in the cells of many body tissues, including the heart, liver, kidneys, skeletal muscle and brain. LDH catalyzes the interconversion of pyruvate and lactate with concomitant interconversion of NADH and
NAD$^+$ (Figure 3.2). Cells that have lost membrane integrity release LDH into the surrounding medium, therefore it can be used as a biomarker for cell damage. In the assay, disappearance of NADH is measured at 340 nm with pyruvate as the substrate for enzyme activity.

![Chemical Structure]

**Figure 3.1 Catalytic action of Lactate Dehydrogenase**

**Procedure:** 6.6 mM NADH and 30 mM sodium pyruvate were prepared in Tris-Cl (0.2 M, pH 7.3). Reaction was initiated with the addition of 50 µL of the test sample and the disappearance of NADH was monitored at 340 nm, for 5 min at an interval of 1 min.

**Calculations:** The percentage of LDH release was calculated as follows:

\[
% \text{ LDH release} = \left( \frac{\text{Activity of LDH in the supernatant}}{\text{Total LDH activity}} \right) \times 100
\]

Where, Total LDH activity is the activity of LDH in the supernatant and the LDH activity measured after complete cell lysis achieved by sonication.
3.2.5. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) colorimetric assay:
The MDCK cells were seeded into a 96-well plates (1 × 10^4 cells/well). Following plating and a
24 hours recovery for the cells to resume exponential growth at 37°C in a humidified incubator
containing 5% CO₂, the cells were subjected to various treatments. Cells of the control group
were cultured in 100 μL of DMEM medium only (without FBS). For oxalate injury, cells were
treated with 1 mM oxalate in DMEM. The effect of the proteins in the presence of oxalate injury
was assessed by adding purified proteins at various concentrations in the presence of oxalate, to
the cells. To verify whether the proteins by themselves contributed to cytotoxicity, cells were
also incubated with the various concentrations of the proteins in the absence of oxalate. After 48
hours of treatment the cytotoxicity of the proteins extracted from the matrix of human calcium
oxalate kidney stone was assessed by MTT colorimetric assay [Lee et al., 2010].

Principle
This is a colorimetric assay that measures the reduction of yellow MTT by mitochondrial
succinate dehydrogenase. The MTT enters the cells and passes into the mitochondria where it is
reduced to an insoluble, coloured (dark purple) formazan product. The cells are then solubilised
with an organic solvent (eg. isopropanol) and the released, solubilised formazan reagent is
measured spectrophotometrically. Since reduction of MTT can only occur in metabolically active
cells the level of activity is a measure of the viability of the cells. The developed colour was read
at a wavelength of 570/630 nm using a micro-plate reader.

Chemicals:
- 5mg/mL MTT in PBS (1X)
- Acid isopropanol: 35μL HCl in 10mL isopropanol
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Procedure:
After 48 hours of treatment, 30 µL of MTT (final concentration of 0.5 mg/mL) was added to each well. Three hours later the supernatant was discarded and acid isopropanol was added to dissolve the formazan crystals. Absorbance values were determined at a 570 nm test wavelength and a 630 nm reference wavelength to test the cell viability using a microplate reader [Karamustafa F et al., 2006].

Calculations:
Cell viability (%) = \frac{(\text{OD value of treated well} - \text{OD value of the blank})}{(\text{OD value of untreated well} - \text{OD value of the blank})} \times 100

3.2.6. Sulforhodamine B (SRB) assay
The MDCK cells were seeded into a 96-well plates (1 × 10^4 cells/well). Following plating and a 24 hours recovery for the cells to resume exponential growth at 37°C in a humidified incubator containing 5% CO₂, the cells were subjected to various treatments. Cells of the control group were cultured in 100 µL of DMEM medium only (without FBS). For oxalate injury, cells were treated with 1 mM oxalate in DMEM. The effect of the proteins in the presence of oxalate injury was assessed by adding purified proteins at various concentrations in the presence of oxalate, to the cells. To verify whether the proteins by themselves contributed to cytotoxicity, cells were also incubated with the various concentrations of the proteins in the absence of oxalate. After 48 hours of treatment the cytotoxicity of the proteins extracted from the matrix of human calcium oxalate kidney stone was assessed by SRB colorimetric assay [Vichai and Kirtikara, 2006]

Principle: The sulforhodamine B (SRB) assay is used for cell density determination, based on the measurement of cellular protein content. Its principle is based on the ability of the protein dye SRB to bind electrostatically and pH dependent on protein basic amino acid residues of trichloroacetic acid-fixed cells. Under mild acidic conditions it binds to and under mild basic
conditions it can be extracted from cells and solubilized for measurement. The optical density of the bound protein stain was read at 490 nm using a micro-plate reader.

Chemicals:
- 40 w/v w/w ice-cold trichloroacetic acid (TCA)
- 0.4% w/v SRB in 1% v/v acetic acid
- 1% v/v acetic acid for washing cells
- 10 mM Tris (hydroxymethyl) aminomethane buffer (TRIS base)

Procedure:
Sulphorhodamine (SRB) assay was performed as described previously by Houghton P [Vichai and Kirtikara, 2006]. After 48 hours of treatment, cells were fixed by means of protein precipitation with 40% ice-cold TCA (50 μL/well, final concentration of 1%) for 1 hour at 4°C. After fixation, cells were washed for five times with distilled water and were stained for 30 min. with 0.4% SRB dissolved in 1% acetic acid (100 μL/well) at room temperature. The unbound SRB dye was removed by washing the plates five times with 1% acetic acid. The plates were air dried and the bound protein stain was solubilised with 200 μL/well of 10 mM unbuffered Tris Base (pH 10.5). The optical density was read at 490 nm.

Calculations:
\[ \% \text{ cell viability} = \left( \frac{At-Ab}{Ac-Ab} \right) \times 100 \]

Where  
- \( At \) = Absorbance value of test compound  
- \( Ab \) = Absorbance value of blank  
- \( Ac \) = Absorbance value of control
3.2.7. Acridine Orange/Ethidium Bromide Staining To Detect Apoptosis:

The MDCK cells were seeded into a 24-well plates (0.5 × 10^5 cells/well). Following plating and a 24 hours recovery for the cells to resume exponential growth at 37°C in a humidified incubator containing 5% CO₂, the cells were subjected to various treatments. Cells of the control group were cultured in 100 μL of DMEM medium only (without FBS). For oxalate injury, cells were treated with 1 mM oxalate in DMEM. The effect of the proteins in the presence of oxalate injury was assessed by adding purified proteins at a concentration of 4μg/ml in the presence of oxalate, to the cells. To verify whether the proteins by themselves contributed to cytotoxicity, cells were also incubated with a concentration of 4μg/mL of the proteins in the absence of oxalate. After 48 hours of treatment the cytotoxicity of the proteins extracted from the matrix of human calcium oxalate kidney stone was assessed by Acridine Orange/Ethidium Bromide Staining.

**Principle:** The ethidium bromide/acridine orange stain (EB/AO stain) is a viability stain that detects apoptotic cells. Viability stains determine the membrane integrity of a cell based on the uptake or exclusion of a dye from the cell. Ethidium bromide is a dye that is only able to pass through the membrane of a dead or dying cell. Acridine orange is a membrane-permeable dye that will stain all cells in the sample. Each dye that is taken up by a cell fluoresces- AO makes a cell green, and EB makes a cell red. Differential staining patterns using a combination of two fluorescent dyes, acridine orange and ethidium bromide, was used to categorise the manner in which cell cytotoxicity was taking place in the cells (Figure 12, 13). Acridine Orange (AO) enters all cells and results in a green appearance of the nuclei. When the cytoplasmic integrity is compromised, ethidium bromide (EB) permeates the cells and stains the nucleus red. This results in an easily distinguishable pattern, wherein live cells have a normal green nucleus; apoptotic cells range from having a bright green to orange nucleus with a condensed and fragmented chromatin; necrotic cells display a structurally normal orange nucleus.
**Materials & Methods**

**Chemicals:**
- Acridine orange (AO)
- Ethidium bromide (EtBr)
- Ethylenediamine-tetraacetic acid (EDTA)
- The dye mix for the EB/AO staining was 100 μg/ml acridine orange and 100 μg/ml ethidium bromide in PBS

**Procedure:**
After 48 h of treatment, the cell suspension from each well along with cells after trypsinization were pooled together into eppendorf vials. The vials were centrifuged at 129 g for 5 min. The pellet obtained was washed with 1X PBS and stained with Acridine Orange/Ethidium Bromide solution of 25 μL PBS and 2 μL EB/AO dye (100 μg/ml). The cells were place on a glass-slide covered with cover-slips and were visualized under the fluorescence microscope at a magnification of 200X. The following parameters were taken into consideration while capturing photographs: Excitation wavelength and Emission wavelength used for acridine orange was 440 nm to 480 nm and 520 nm to 560 nm, while Excitation wavelength and Emission wavelength for ethidium bromide was 445 nm and 605 nm [Ribble et al., 2005].

**3.2.8. Statistical analysis**
The experiments were performed in triplicate. The data are presented as means ± SD. Statistical evaluation was conducted by one-way ANOVA using Tukey's multiple comparison. A minimum P value < 0.05 was considered to be the minimum level of statistical significance. The software used for analysis was GraphPad InStat.
3.3. SDS PAGE

3.3.1. 1-D SDS PAGE
For SDS-PAGE, protein samples were lyophilized and reconstituted in reducing sample buffer and analyzed by one-dimensional discontinuous SDS-PAGE using 1 mm thick, 10% separating and 4.4% stacking gels with a Mini-Protean III apparatus (Bio-Rad). Protein bands were stained with silver using ProteoSilver™ Plus Silver Stain Kit (Sigma-Aldrich Co.). Broad range molecular weight markers (Bio-Rad) were used as standards [Kaur et al., 2009].

- 30% Stock Acrylamide
  - 73g Acrylamide
  - 1.95g Bis Acrylamide
  - Make upto 250 ml with distilled water

- Sample Buffer
  - 0.72g Tris Base
  - 6.23g Glycerol
  - 2.5mg Bromophenol Blue
  - 2.5 g SDS
  - Make upto 25 ml with distilled water for 5X or to 125ml for 1X

- Running Buffer (pH = 8.3 – 8.7)
  - 3* 202g Tris Base
  - 10* 288g Glycine
  - 2*100g SDS
  - Make upto 20L with Distilled Water

- Lower Gel Buffer (pH = 8.8)
  - 45.43g Tris Base
  - 5.75ml Conc. HCl
  - 1g SDS
  - Make upto 250ml with distilled Water
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- **Upper Gel Buffer** (pH = 6.8)
  - 7.58g Tris Base
  - 4.8ml Conc. HCl
  - 0.5g SDS
  - Make upto 125ml with distilled Water

- **Ammonium Persulfate (APS)**
  - 100mg per ml of distilled water

- **Coomassie Blue Stain and Fixative**
  - 500ml Methanol
  - 400ml Distilled Water
  - 100ml Glacial Acetic Acid
  - 1g Coomassie Brilliant Blue R-250

- **Destain**
  - 300ml Ethanol
  - 2.49ml Distilled water
  - 210ml Glacial Acetic Acid

3.3.2. 2-D SDS PAGE
The samples were desalted using ReadyPrep 2-D Cleanup Kit and dissolved in 125 µl of sample rehydration buffer containing 8 M urea, 2% w/v CHAPS, 50 mM DTT, 0.2% w/v Ampholytes and 0.0002% bromophenol blue. IEF was first carried out using Bio-Rad IPG strip (pH 3–9; 7 cm) in Bio-Rad protean IEF cell according to manufacturer’s instructions, followed by equilibration for 15 min each in equilibration buffer I (6 M Urea, 2% SDS, 0.375 M Tris HCl (pH 8.8), 20% Glycerol, 130mM DTT) and equilibration buffer II (6 M Urea, 2% SDS, 0.375 M Tris HCl (pH 8.8), 20% Glycerol, 135mM Iodoacetamide). Equilibrated IPG strips were loaded onto a 10% polyacrylamide gel sealed with overlay agarose, and electrophoresed at a constant voltage of 100 V. The gel was stained by silver staining and analysed using Biorad PD Quest
Materials & Methods

Advanced 2D Analysis Software. The spots of interest were manually excised from the gel and were destained using destainer provided in the ProteoSilver™ Plus Silver Stain Kit followed by in-gel digestion using Trypsin profile IGD kit. The proteins were identified by matrix assisted laser desorption/ionization-time of flight (MALDI-TOF) MS followed by MASCOT database search. [Aggarwal et al., 2013b].

3.4. Purification of potent protein

3.4.1. Materials

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

3.4.2. Extraction of proteins

Only calcium 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. Around 120 gm 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 10000 rpm for 20 minutes at 4°C. Supernatant was separated into >3 kDa and <3 kDa fractions by centrifugation with the help of Amicon Ultra centrifugal separating tubes (Millipore) of 3 kDa cut off molecular weight.

3.4.3. Protein estimation

Protein concentration of whole EGTA extract, >3 kDa and <3 kDa fraction was estimated by Lowry assay [Lowry et al., 1951].
3.4.4. Ion exchange chromatography

3.4.4.1. Sample preparation

The >3 kDa fraction which exhibited activity against calcium oxalate nucleation and growth assay system was used for further analysis. The sample obtained was lyophilized and was preserved at -80°C.

3.4.4.2. Preparation of column

- **Washing of slurry**

Macro Prep 25 Q strong anion exchanger support and Macro Prep 25 S strong cation exchanger support was supplied hydrated in 20% (v/v) ethanol respectively. 100 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 (20 mM TrisCl +0.1 M NaCl) for Macro Prep 25 Q strong anion exchanger 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. Similarly, procedure was followed for Macro Prep 25 S strong cation exchanger with buffer A (20 mM Mops +0.1 M NaCl).

- **Column packing**

One third of column (50X1 cm) 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.
3.4.4.3. Sample loading & separation of biomolecules

Lyophilized sample was reconstituted with starting buffer and was loaded into the column. The bound protein was eluted with 0-90% of linear gradient of buffer A and B. For anion exchanger 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). For cation exchanger buffer A consists of 20mM Mops with 0.1M NaCl (pH 7.4) and Buffer B consists of 20mM Mops with 1.0 M NaCl (pH 7.4). Protein elution was done at flow rate of 0.5 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 and cation exchange chromatography is illustrated in table 3.1.

Fractions coming under the peak were pooled and dialyzed. Their activity was checked on calcium oxalate nucleation and growth crystal assays followed by 1-D SDS-PAGE run. Fraction which was showing highest activity and few bands was then lyophilized and was preserved for further purification.

<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>0.5</td>
<td>Buffer A</td>
</tr>
<tr>
<td>25-65</td>
<td>0.5</td>
<td>0-90% gradient of buffer A to buffer B</td>
</tr>
<tr>
<td>65-85</td>
<td>0.5</td>
<td>Buffer B</td>
</tr>
<tr>
<td>85-155</td>
<td>0.5</td>
<td>Buffer A</td>
</tr>
</tbody>
</table>

Table 3.1 Method used for anion/cation exchange chromatography
3.4.5. Molecular sieve chromatography

3.4.5.1. 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.

3.4.5.2. Column packing
Column packing was done by using the same method as described in section 3.4.4.2. column packing for ion exchange chromatography.

3.4.5.3. Sample loading & separation of biomolecules
Fraction showing activity was concentrated and loaded on a Bio gel® P-100 gel molecular sieve column(30X1cm) 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 nucleation and growth assay system. Also their activity was tested on oxalate injured Madin Darby Canine Kidney (MDCK) renal epithelial cells. Potent fractions thus obtained were subjected to 1-D SDS-PAGE and reverse phase HPLC (RP-HPLC).

<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>Buffer</td>
</tr>
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</table>

Table 3.2 Method used for molecular sieve chromatography
3.4.6. 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 [Aggarwal et al., 2013c].

3.4.7. 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 ProteoSilver™ Plus Silver Stain Kit. Trypsin profile IGD kit 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.

3.4.8. 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-hydroxycinnamicacid) 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, NCBInr) 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.

3.5. In silico study

3.5.1. Materials
Molecular operating environment (MOE) 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.

3.5.2. Homology modeling and structure validation:
Homology model building of the identified proteins was done using molecular operating environment (MOE) [Pathak et al., 2011, Latha et al. 2006]. The template used for model building of the identified proteins was based upon the similar structures found on Protein Data Bank. The structure obtained were energy minimized using OPLS 2005 force field with Polak-Ribiere Conjugate Gradient (PRCG) algorithm. The minimization was stopped either after 5,000 steps or after the energy gradient converged below 0.001 kcal/mol. All atom molecular dynamics (MD) simulation of protein structures in explicit water was carried out using the GROMACS 4.5.4 software [Lai and Crasto, 2012] and the GROMOS96 force field for a time scale of 10 ns. Three-dimensional periodic boundary conditions were imposed, enclosing the molecule in a dodecahedron solvated with the SPC216 water model provided in the GROMACS package and energy minimized using 1000 steps of steepest descent. The LINCS [Hess et al., 1997] algorithm
was used to constrain all bond lengths and cut-off distances for the calculation of the coulombic and van der Waals interactions at 1.0 nm. The system was equilibrated by 100 ps of MD runs with position restraints on the protein to allow the relaxation of the solvent molecules at 300 K and normal pressure. The overall quality of the model, stereochemical values and non-bonded interactions were tested using PROCHECK [Laskowski et al., 1993], ERRAT [Colovos et al., 1993] and VERIFY3D [Eisenberg et al., 1997].

3.5.3. Docking of Homology model of protein 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. Binding sites of proteins were predicted by using active site finder tool of MOE software. Molecular docking of calcium oxalate was done using MOE-Dock. MOE-dock utilizes a Monte Carlo Simulated Annealing (SA) method in docking calculations to search for favorable binding configurations of a small, flexible ligand and a rigid macromolecule in a pre-set box. The docking energy calculation was carried out within a user-specified three-dimensional docking box (3D docking box) using the simulated annealing method and OPLS-AA force field. The energy grids for docking were generated as grid-based potential fields by the MOE-Dock program. Docking energy was calculated as the sum of the electrostatic, Van der Waals, and flexibility energies. The Van der Waals parameters were taken from the force field. The electrostatic field was calculated based on force field in the Coulombic manner using the constant dielectric of 1.0 for solvation. MOE-Dock performed 25 independent docking runs and the lowest docking energy conformation for each binding site was chosen for LIGPLOT analysis [Gul et al., 2007].

3.5.4. Molecular interaction of calcium oxalate proteins:
To investigate the precise interaction between calcium oxalate and proteins identified in this study we have mutated the amino acids in the binding site. Precisely all the acidic and basic amino acids were mutated to alanine and the amino acids such as Tyrosine, Threonine and Serine
in the active site were phosphorylated. After incorporating these mutations, calcium oxalate was docked into the binding site using MOE-Dock with similar parameter as was used for wild type.