RESULTS
4.1 Comparison of EGTA, SDS and acetic acid extraction methods for protein extraction from renal calculi

Three different extraction methods were used for extraction of proteins from the renal calculi. This study was done to compare different methods of protein extraction from COM stones to investigate the soluble matrix proteins involved in the formation of calcium oxalate monohydrate (COM) stone.

4.1.1. Protein concentration

Maximum amount of protein (0.64mg/mL) was extracted by using SDS method followed by 0.24mg/mL and 0.10mg/mL by EGTA and acetic acid extraction methods respectively. Figure 4.1 shows the concentration of proteins extracted by SDS, EGTA and acetic acid extracts respectively.

4.1.2. Effect of EGTA, SDS and Acetic extract on calcium oxalate (CaOx) crystal growth

Though the amount of protein extracted by SDS was high but its activity on CaOx crystal growth system was minimal. EGTA extract exhibited highest inhibitory activity on CaOx crystal growth out of the three extracts. Only 2.64% of inhibitory activity was shown by the SDS extract, while 6.47% by acetic acid which is insignificant when compared to the EGTA extraction process. % inhibition by different extraction methods is shown in table 4.1. Though, the amount of protein extracted by EGTA method was 0.24mg/mL but its activity on CaOx crystal growth was quite promising. Therefore, EGTA extraction method was selected for the renal stone extraction to carry out the further studies.
4.1.3. SDS-PAGE

SDS-PAGE analysis of three different extracts is shown in the Figure 4.2. Maximum numbers of protein bands were visible in SDS extract while few were visible in EGTA and acetic acid extracts which is in conformity with the amount of proteins extracted by SDS, EGTA and acetic acid extraction methods respectively (Figure 4.1).

![Graph showing amount of proteins extracted](image)

**Figure 4.1.** Amount of proteins extracted by SDS, EGTA and acetic acid. SDS extracted maximum protein followed by EGTA and acetic acid.

**Table 4.1** Percentage inhibition exhibited by different extract on CaOx growth assay system. EGTA extract is exhibiting highest inhibitory activity among all extracting methods.

<table>
<thead>
<tr>
<th>Buffer</th>
<th>% inhibition at 720 sec</th>
</tr>
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<tbody>
<tr>
<td>EGTA</td>
<td>98%</td>
</tr>
<tr>
<td>SDS</td>
<td>2.64%</td>
</tr>
</tbody>
</table>
Figure 4.2. SDS-PAGE analysis of three different extract. First lane is showing the molecular weight marker while second, third and fourth is showing the bands of SDS, EGTA and acetic acid extract simultaneously. Maximum protein bands were visible in SDS extract while few were visible in EGTA and acetic acid extract.

4.2. Effect of lipid extract of renal stones on CaOx crystal growth

Different volumes of lipid extract were tested on CaOx crystal growth system. From Figure 4.3 it was observed that lipid extract did show an inhibition towards CaOx crystal growth however, with the increase in the volume of lipid extract inhibition decreases.
**Figure 4.3.** Effect of various volumes of lipid extract on CaOx crystal growth. Percentage inhibition of calcium oxalate growth by various volumes of lipid extract.

### 4.3. Effect of whole extract, >10 kDa and < 10kDa fractions on CaOx crystal growth system

Both high as well as low molecular weight biomolecules are reported to play role in kidney stone formation therefore, for this purpose EGTA extract was separated into >10kDa and <10kDa fractions. Since, EGTA extract was able to extract optimum amount of protein and also exhibited bioactivity therefore, it was selected for further investigations on calcium phosphate (CaP) mineralization system as well as on calcium oxalate crystal growth assay system.
Activities of all the three EGTA fractions obtained after filtering through Amicon ultra centrifugal filter device with a molecular weight cut off 10 kDa were studied on CaOx crystal growth assay system. Effect of various volumes of renal extract can be depicted from the graph shown in Figure 4.4. It was found that whole EGTA extract was showing highest percentage of inhibition. Whole EGTA extract (239.46 μg/mL protein) as well as its >10 kDa fraction (154.78 μg/mL protein) exhibited significant inhibitory activity as compared to the <10 kDa fraction (72.60 μg/mL protein).

![Graph showing inhibition percentages](image)

**Figure 4.4** Percentage inhibition of whole EGTA extract, >10kDa and <10kDa fractions on CaOx crystal growth system.

4.4. **Effect of whole extract, >10 kDa and < 10kDa fractions from human renal matrix of calcium oxalate monohydrate stones on in vitro calcium phosphate mineralization**

Three phases of CaP mineralization viz. initiation, growth and demineralization were assessed by the whole EGTA extract, >10 kDa and < 10kDa fractions.
4.4.1. Effect of whole extract, >10 kDa and < 10kDa fractions on initial mineral phase of CaP assay system

Figure 4.5 depicts the effect of EGTA extract fractions on initial mineral phase formation. It is evident from the Figure 4.5a that 98.97% of phosphate ion inhibition was exhibited by whole renal stone extract, 85.9% by >10kDa and 92.09% by <10kDa fraction on in vitro homogenous assay system of calcium phosphate. However, Figure 4.5b shows that the whole extract showed maximum 81.64% of calcium ion inhibition. Interestingly, both type of activity stimulatory (maximum 25.2%) as well as inhibitory activity (maximum 25.01%) was shown by >10kDa fraction. <10kDa fraction showed 96.23% of maximum inhibition.

4.4.2. Effect of whole extract, >10 kDa and < 10kDa fractions on growth of preformed mineral phase

Figure 4.6 shows the percentage inhibition or stimulation of phosphate and calcium ions on growth of preformed mineral phase. From the figure, it could be inferred that inhibition of phosphate ions increased with the increase of whole extract volume. >10kDa fraction stimulated the growth of phosphate ions on preformed mineral phase. While <10kDa showed both types of activities (Figure 4.6a).

Inhibition of calcium ions was shown by whole extract and <10kDa fraction on the growth of preformed mineral phase. Stimulation was seen by various volumes of >10kDa fraction (Figure 4.6b). From the Figure, it is evident that whole EGTA extract is exhibiting maximum inhibitory potency towards phosphate ions but its activity is low towards calcium
ion inhibition. In addition, >10 kDa fraction showed maximum stimulatory activity against the growth of calcium ions but minimum stimulatory activity against phosphate ions growth on preformed mineral phase.

4.4.3. Effect of whole extract, >10 kDa and < 10kDa fractions on demineralization of preformed mineral phase

Release of phosphate ions increased with the increase of volume of different fractions. Figure 4.7a shows maximum amount of release of phosphate ions with whole extract. On the other hand, percentage release of calcium ions was decreased with the increase of different extract volumes. Low volume of whole extract exhibited the maximum ability to demineralize calcium ions from preformed mineral phase as is clearly depicted in Figure 4.7b. Among all the three fractions whole extract showed highest percentage of release of phosphate ions followed by <10kDa and >10kDa fractions.

It could be inferred that whole EGTA extract exhibited inhibitory activity in initial and growth mineral phase. Interestingly, stimulatory as well as inhibitory activity towards initial mineral phase was shown by >10 kDa fraction. Stimulatory activity was retained in growth mineral phase by this fraction. On the other hand, inhibitory activity was shown by <10kDa in initial mineral phase. Moreover, both type of activity was shown by <10kDa fraction in the case of phosphate ions but an inhibition was observed in the case of calcium ions on preformed mineral phase.

High percentage of phosphate ion was released with high volume of all the three fractions. But the opposite trend was observed with calcium ion demineralization. It was
found that high percentage of calcium ion was released with low volume of all the three fractions.

Figure 4.5 a

![Graph showing inhibition/stimulation of phosphate ions with volume of extract]

Figure 4.5 b
**Figure 4.5** Effect of various volumes of renal stone extract (whole extract, >10kDa, <10kDa) on initial mineral phase. Figure 4.5 a. Percentage inhibition/stimulation of phosphate ions and calcium ions (Figure 4.5 b) by whole EGTA extract, >10kDa and <10kDa fractions different renal stone extracts.

**Figure 4.6 a**
Figure 4.6 b

Figure 4.6 Percentage inhibition or stimulation of phosphate ions (a) and calcium ions (b) by different volumes of whole EGTA extract, >10kDa and <10kDa fractions of renal stone extract on the growth of preformed mineral phase.

Figure 4.7 a
Figure 4.7 Effect of various volumes of renal stone extract (whole extract, >10kDa, <10kDa) on demineralization of preformed mineral phase. Percentage of phosphate ions (a) and calcium ions (b) demineralized by different fractions of renal stone extract.
4.5. Purification and identification of potent protein

More than 10kDa fraction exhibited maximum activity towards calcium oxalate crystal growth assay system and hence was loaded on a strong anion exchanger Macro Prep® 25 Q column. Consecutive fractions were collected with increasing gradient, pooled and were named fraction P1 to P7 (Figure 4.8). It was found that P6 fraction exhibited highest inhibitory activity against CaOx crystal growth and its SDS-PAGE analysis showed presence of few bands (Figure 4.9 & Figure 4.10). This fraction was further purified by molecular sieve chromatography on a Bio gel® P-100 gel molecular sieve column (50 X 1cm).

Proteins were eluted at pH 7.4 with 20 mM Tris buffer. Fractions were collected and pooled as P1’, P2’, P3’, and P4’ (Figure 4.11) and examined for inhibitory activity against CaOx crystal growth. It was found that fraction P2’ had a relatively strong inhibitory activity (Figure 4.12). All the three fractions were analyzed further by 12% SDS-PAGE and pooled fraction P2’ showed the presence of a single band having molecular weight ~ 42 kDa indicating that a novel CaOx crystal growth inhibitor from human renal stone matrix protein was purified (Figure 4.13).

The homogeneity of fraction P2’ obtained after molecular-sieve chromatography was confirmed on RP-HPLC which showed a single peak at a retention time of ~9 minutes (Figure 4.14).
Figure 4.8 Elution profile of protein sample loaded on anion exchanger. Seven fractions (P1 to P7) were collected with a linear gradient of NaCl.
**Figure 4.9** Characterization of inhibitory activity of various fractions after anion exchange chromatography. Fraction P6 showed highest inhibitory activity against CaOx crystal growth.

**Figure 4.10** SDS-PAGE of pooled fractions after anion exchange chromatography
Figure 4.11 Elution profile of fraction P6 loaded on molecular sieve chromatography column after anion exchange chromatography. The eluting proteins were detected at 280 nm. Shaded portion (fraction P2') indicate elution of most active fraction having highest inhibitory activity.
Figure 4.12 Percentage activity of pooled fractions of molecular sieve chromatography. P2’ fraction is showing maximum inhibitory activity.

Figure 4.13 SDS-PAGE of fraction P2’ after molecular sieve chromatography. A single band of MW~ 42kDa is shown by an arrow.
Figure 4.14 Homogeneity and molecular mass determination of protein from molecular sieve chromatography having highest CaOx inhibitory activity. RP-HPLC of fraction P2’ for homogeneity, showing a single peak at 9 min.

4.5.1. Mass spectrometric identification of human phosphate cytidylyltransferase 1

The protein band detected in fraction P2’ was excised, in-gel tryptic digested and identified by matrix assisted laser desorption/ionization–time of flight (MALDI-TOF) MS. The MALDI-TOF-MS spectrum of purified protein P2’ is shown in Figure 4.15. Using the Mascot search engine (http://www.matrixscience.com), the MALDI-TOF data obtained from fraction P2’ was matched significantly with human phosphate cytidylyltransferase 1, choline, beta [Homo sapiens]. The matching score (Figure 4.16) of mascot search was 38 and sequence coverage was 17%. Phosphate cytidylyltransferase 1 (gi|21361202) catalyzes the condensation of CTP and phosphocholine to form CDP-choline as the rate-limiting and regulatory step in the CDP-choline pathway.
Figure 4.15 Identification of novel protein by MALDI-TOF MS. Peptide mass fingerprinting by MALDI-TOF MS obtained from trypsinized fraction P2'. 
Mascot Search Results

Protein View

Match to: AAD35088 Score: 38 Expect: 21
AF148464 NID: - Homo sapiens

Nominal mass (M): 42199, Calculated pI value: 5.99
NCBI BLAST search of AAD35088 against nr
Unformatted sequence string for pasting into other applications

Taxonomy: Homo sapiens
Links to retrieve other entries containing this sequence from NCBI Entrez:
PCY1B_HUMAN from Homo sapiens

Fixed modifications: Carbamidomethyl (C)
Variable modifications: Oxidation (M)
Cleavage by Trypsin: cuts C-term side of KR unless next residue is P
Number of mass values searched: 24
Number of mass values matched: 11
Sequence Coverage: 17%

Matched peptides shown in Bold Red

1 MPVVTTDAES ETGIPKSLSN EPPSETMEEI EHTCPQPRLT LTAPAPFADE
51 TNCQCAPEKLTLAQARLG TPADRPVRVY ADGIFDLFHS GHARALMQAK
101 TLFPSNYLLV GVCSSDLTHK FKGFTVMNEA ERYEALRHCR YYDEVIRDA
151 WTLTPEFLEK HKIDFVAHDD IPIYSSAGSDD VYKHIEAGM FVPTQRTEGI
201 STSDIITRIV RDYDVYAR RN LQRGYTAKE LNVSFINERKY RFQNPQVDMK
251 EKVKNVEERS KEFVNREVEEK SHDLIQK WEE KSREEFIGNFL ELFGPDPGAWK
301 QMFQERS SRRM LQALSPQKQP VSSPTRSRSP SRSPSPTFSW LPLKTSPPSS
351 PKAASASISS MSEGDEDEK

Figure 4.16 Mascot search result showed that purified protein is similar to Phosphate cytidylyltransferase 1 choline beta [Homo sapiens]
4.6. In silico study

4.6.1. Homology modeling of human CCT1 choline beta and docking with calcium oxalate

The protein phosphate cytidylyltransferase 1, choline, beta (CCT) was homology modeled based on sequence and structure alignments with the chain A CTP: glycerol-3 phosphate cytidylyltransferase (GCT) of Bacillus subtilis (Protein Data Bank IC 1 COZ-A) (Fig 4.17a and 4.17b). Three binding sites were predicted by the MOE site finder in the wild type CCT and were named site1, site2 and site3. Calcium oxalate was docked within the active site using the Monte Carlo docking procedure of MOE and repeated cycles of protein and substrate minimization.

a. X-ray crystallographic structure of GCT  
b. Homology model of CCT used as template.
**Figure. 4.17** X-ray crystallographic structure of GCT was used as a template (fig. a) for homology modelling. (Fig. b) showing homology model of CCT.

During the initial stage of the docking procedure, the side chains of the protein are fixed. The best-ranking docking modes of the ligands are identified and energy minimized in the protein, while allowing full side chain flexibility. Docking of calcium oxalate with all the three binding sites gave best docking score of -59.8251, -56.4486 and -106.304 with site1, site2 and site3 respectively (Table 4.2a).

LIGPLOT analysis of the binding site reveals involvement of different amino acids of protein with calcium oxalate. In site1 (first binding site) glycine, histidine, alanine, asparatic acid, glutamine, tyrosine and aspartic acid at 16, 17, 20, 94, 120, 138 and 139 positions were involved in interaction with calcium oxalate (Figure 4.18a). In the site 2, methionine at 22 and tyrosine at 66 positions were found to be participating in the interaction with calcium oxalate (Figure 4.18b). Aspartic acid, lysine and alanine at 41, 45 and 74 positions respectively in site3 interacted with calcium oxalate. In this binding site, most of the atoms of calcium oxalate are highly exposed. Aspartic acid at position 41 is covalently bound to the calcium of the ligand (calcium oxalate). Lysine at position 45 is bound to the oxygen, where it is acting as side chain receptor (Figure 4.18c).
In all of these active sites of the wild type modeled protein human CCT 1 choline beta, it was observed that acidic amino acids played a significant role in the interaction with calcium oxalate.

Figure 4.18 Ligplot showing interaction of various amino acids present in binding site 1 (Figure a), site 2 (Figure b) and site 3 (Figure c). Aspartate at position 94 & 139 is
interacting with calcium oxalate in binding site 1 while in binding site 3 it is binding with calcium. Amino acids tyrosine and lysine is binding with oxygen of calcium oxalate in binding site 2 and site 3 respectively.

From Ramachandran plot (Figure 4.19) it was found that out of 369 amino acids of CCT, 329 were in red region (89.15%) and 32 (8.67%) in yellow region and 8 (2.1%) was in outer region. This result indicates that the quality of the modeled protein was highly reliable.

Figure 4.19 Ramachandran plot showing maximum amino acids in red region.

4.6.2. Point mutation of acidic amino acid of active site and docking with calcium oxalate
Upon substitution of acidic amino acids present in the active sites (site 1 and site 3) of the wild type modeled protein CCT with alanine, glycine, lysine, arginine and histidine, positive docking scores were obtained indicating a poor interaction with calcium oxalate. Since, no acidic amino acid was present in the binding site 2 therefore, it was not mutated. No docking score was found when acidic amino acid present in binding site 1 was point mutated with arginine and histidine (Table 4.2b). In all of these active sites of the wild type modeled protein CCT, it was observed that acidic amino acids played a significant role in the interaction with calcium oxalate.

Table 4.2 Table (a) showing docking score of wild type CCT and table (b) of mutated type. Negative value of wild type is indicating good binding with CaOx while positive value showing poor binding.

Table 4.2a

<table>
<thead>
<tr>
<th></th>
<th>Site1</th>
<th>Site2</th>
<th>Site3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type human</td>
<td>-59.8251</td>
<td>-56.4486</td>
<td>-106.3040</td>
</tr>
<tr>
<td>CCT</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 4.2b

<table>
<thead>
<tr>
<th>Point mutation of CCT with amino acids A,G,K,R,H</th>
<th>Site1</th>
<th>Site2</th>
<th>Site3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alanine(A)</td>
<td>313.2496</td>
<td></td>
<td>7.3765</td>
</tr>
<tr>
<td>Glycine(G)</td>
<td>370.3134</td>
<td></td>
<td>257.1444</td>
</tr>
<tr>
<td>Lysine(K)</td>
<td>494.8806</td>
<td></td>
<td>7.8061</td>
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<tr>
<td>Arginine (R)</td>
<td></td>
<td></td>
<td>634.8440</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Histidine (H)</td>
<td>–</td>
<td>–</td>
<td>343.5878</td>
</tr>
</tbody>
</table>

These studies clearly demonstrate that acidic amino acids present in the wild type CCT showed a good docking score, an indicator of interaction with calcium oxalate, while substitution of these acidic amino acids with alanine, glycine, lysine, arginine and histidine gave a poor docking score. (Table 4.2b)