Results
3.1. In vitro antilithiatic activity of plants

The efficacy of *Trachyspermum ammi* was compared with other known antilithiatic plants like *Zingiber officinale* and *Rubia cordifolia* using *in vitro* homogenous assay system for initiation, growth and demineralization of calcium phosphate mineralization. To evaluate the effectiveness of the three plants an equal concentration (10% w/v) of aqueous extract of all three plants was prepared to test *in vitro* calcium phosphate (CaP) mineralization.

3.1.1. Comparative analysis of three plants on initiation of calcium phosphate (CaP) mineral phase

The crude aqueous extract (10% w/v) of all the three plants was tested for its ability to inhibit initiation of CaP mineral phase at varying volumes. Figure 3.1 shows the percentage inhibition of CaP mineral phase by 0.25 ml, 0.50 ml, 0.75 ml, 1.00 ml and 1.25 ml of all plants extract. From the figure 3.1a it can be observed that out of the three plants *Trachyspermum ammi* and *Rubia cordifolia* are showing highest and comparable percentage inhibition of calcium ions precipitation, which is about 82.14% by *Trachyspermum ammi* and 80.07% for *Rubia cordifolia* by 1.25 ml of aqueous extract. Among all three plants *Zingiber officinale* showed minimum percentage of inhibition of calcium ions (49.95%). Similarly, in the case of phosphate ions precipitation (Figure 3.1b) similar trend is observed, *Trachyspermum ammi* and *Rubia cordifolia* are showing comparable and higher inhibitory potency (80.04% and 79.05% respectively) whereas *Zingiber officinale* is showing a maximum percentage of inhibition at 1.25 ml i.e. about 53.85%. In the case of *Rubia cordifolia*, it was observed that when the volume of extract is 0.5 ml, the percentage of inhibition remained almost constant till 1.25 ml of extract, whereas for *Trachyspermum ammi* the percentage of inhibition for both Ca$^{2+}$ and HPO$_4^{2-}$ ions increased linearly till 1.25 ml of extract. On the other hand, *Zingiber officinale* extract showed very little tendency to inhibit CaP mineral phase initiation, for both Ca$^{2+}$ and HPO$_4^{2-}$ ions below the volume of 1.25 ml.
**Figure 3.1a.** Percentage inhibition of calcium ions by three plant extracts

**Figure 3.1b.** Percentage inhibition of phosphate ions by three plant extracts

**Figure 3.1.** Comparative analysis of three plants towards initiation of CaP mineral phase formation (Values are mean ± SD, n = 5)
3.1.2. **Comparative analysis of three plants on growth over CaP preformed mineral phase**

The activity of aqueous extract of all the three plants to inhibit growth of preformed mineral phase is graphically represented in figure 3.2. From the graph, it is evident that *Trachyspermum ammi* has maximum potential to inhibit growth over preformed mineral phase of CaP in comparison to other two plants. The 0.25 ml of its aqueous extract showed 20.09% and 20.03% of inhibition of Ca$^{2+}$ and HPO$_4^{2-}$ ions respectively and further proceeding to 69.15% and 61.12% inhibition for Ca$^{2+}$ and HPO$_4^{2-}$ ions by 1.25 ml of its extract.

Followed by *Trachyspermum ammi*, *Rubia cordifolia* showed a moderate percentage of inhibition of about 40.04% and 42.7% (Ca$^{2+}$ and HPO$_4^{2-}$ ions respectively) at 1.25 ml of extract. Alternatively, *Zingiber officinale* showed minimum inhibitory potency towards growth of CaP mineral phase. Volume of its extract below 1.25 ml showed a minimal percentage of inhibition (7.1% and 6.7% for Ca$^{2+}$ and HPO$_4^{2-}$ ions respectively, at 1.00 ml). The maximum percentage inhibition at 1.25 ml of this extract is 17.9% and 15.8% for Ca$^{2+}$ and HPO$_4^{2-}$ ions respectively.

3.1.3. **Comparative analysis of three plants on demineralization of CaP preformed mineral phase**

The comparative analysis of all three plants towards demineralization of CaP is shown in figure 3.3. The ability of aqueous extract of *Trachyspermum ammi* to demineralize preformed CaP mineral phase was found to be followed by *Rubia cordifolia* and *Zingiber officinale* presented the minimum ability to demineralize the preformed mineral phase among all.

From the figure 3.3, it is obvious that *Trachyspermum ammi* possessed maximum ability to demineralize both Ca$^{2+}$ and HPO$_4^{2-}$ ions from the CaP mineral phase. The 0.25 ml of its aqueous extract has the ability to demineralize about 14.6% of Ca$^{2+}$ ions and 17.4% of HPO$_4^{2-}$ ions.
Results

In vitro antilithiatic activity of plants

Figure 3.2a. Percentage inhibition of calcium ions by three plant extracts

Figure 3.2b. Percentage inhibition of phosphate ions by three plant extracts

Figure 3.2. Comparative analysis of three plants towards growth of CaP mineral phase formation (Values are mean ± SD, n = 5)
Results

In vitro antilithiatic activity of plants

Figure 3.3a. Percentage demineralization of calcium ions by three plant extracts

Figure 3.3b. Percentage demineralization of phosphate ions by three plant extracts

Figure 3.3. Comparative analysis of three plants towards demineralization of CaP preformed mineral phase (Values are mean ± SD, n = 5)
As the volume of aqueous extract increase to 0.50 ml, there is a rapid increase in percentage demineralization of Ca\textsuperscript{2+} and HPO\textsubscript{4}\textsuperscript{2-} ions (44.5% and 48.26% respectively). At 1.25 ml of its aqueous extract, the ability to demineralize Ca\textsuperscript{2+} ions is 62.15% and HPO\textsubscript{4}\textsuperscript{2-} ion is 61.21% from the preformed mineral phase. Although both *Rubia cordifolia* and *Zingiber officinale* showed comparable percentage of Ca\textsuperscript{2+} and HPO\textsubscript{4}\textsuperscript{2-} ions demineralization by 1.25 ml of their respective aqueous extracts, but still the demineralization caused by *Zingiber officinale* aqueous extract below 1.25 ml is very low as compared to that of *Rubia cordifolia*.

Even though, both *Rubia cordifolia* and *Trachyspermum ammi* presented comparable percentage inhibition of initiation of CaP mineral phase (Figure 3.1), but the competence of *Trachyspermum ammi* aqueous extract to inhibit growth and cause demineralization of pretormed mineral phase was adequately higher in comparison to all the three plants. On the other hand *Zingiber officinale* showed very low potential to inhibit CaP initiation and growth as well as demineralization of CaP at low extract volume. So, from this comparison, *Trachyspermum ammi* was finally considered as plant having higher inhibitory potential towards CaP mineral phase as compared to *Rubia cordifolia* and *Zingiber officinale*. Thus, the aqueous extract of *Trachyspermum ammi* was further tested for its ability to inhibit calcium oxalate crystal growth.

### 3.1.4. Effect of *Trachyspermum ammi* aqueous extract on calcium oxalate crystal growth

The efficacy of aqueous extract of *Trachyspermum ammi* was evaluated on calcium oxalate crystal growth assay system. The inhibition of CaOx crystal growth by *Trachyspermum ammi* was evaluated using varied volumes of its aqueous extract (5 \( \mu l \), 10 \( \mu l \), 15 \( \mu l \) and 20 \( \mu l \)). Figure 3.4 shows the percentage inhibition of CaOx crystal growth over its nidus by *Trachyspermum ammi* aqueous extract. It was found that 5 \( \mu l \) of aqueous extract has a very low percentage inhibition of about 7.8%. As the volume of aqueous extract increase from 5 \( \mu l \) to 10 \( \mu l \), the inhibition increased to 33.2%. On the other hand, an increase in volume of aqueous extracts from 10 \( \mu l \) to 15 \( \mu l \) and then to 20
\( \mu l \), showed a gradual increase in inhibition of CaOx crystal growth. The maximum inhibition 43.2\% is exhibited by 20 \( \mu l \) of sample.

![Graph showing percentage inhibition of calcium oxalate crystal growth vs volume of extract (\( \mu l \)).](image)

**Figure 3.4.** Percentage inhibition of calcium oxalate crystal growth by *Trachyspermum ammi* aqueous extract

Further the type of biomolecules in the seeds of *Trachyspermum ammi* possessing antilithiatic activity were identified by partial fractionation based on the molecular weight of biomolecules in the aqueous extract.

**3.1.5. In vitro antilithiatic activity of more than 10 kDa and less than 10 kDa fraction of *Trachyspermum ammi* aqueous extract**

The qualitative identification of biomolecules having antilithiatic property in the seeds of *Trachyspermum ammi* was executed after fractionation of aqueous extract of *Trachyspermum 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 concentration of both these fractions was made equal to concentration of crude aqueous
extract i.e. 10% w/v. Further, the activity of both more than and less than 10 kDa fractions was estimated for their ability to inhibit initiation of CaP mineral phase formation as well as calcium oxalate crystal growth.

Figure 3.5 shows the inhibition of initiation of CaP mineral phase formation by the fraction of aqueous extract of *Trachyspermum ammi* having more than 10 kDa molecular weight biomolecules. The inhibitory activity by 0.25 ml of more than 10 kDa fraction is 84.44% and 84.01% for Ca$^{2+}$ and HPO$_4^{2-}$ ions respectively. On increasing the volume of this fraction further to 0.5, 0.75, 1.0 and finally to 1.25 ml showed a mild increase in its activity. The volume, 1.25 ml of more than 10 kDa extract presented an inhibition of 91.38% of Ca$^{2+}$ and 90.25% of HPO$_4^{2-}$ ions.

On comparing this percentage of inhibition of initial mineral phase with the percentage of inhibition caused by crude extract of *Trachyspermum ammi* (Figure 3.1), it is noticeable that 0.25 ml of crude extract has a much lower inhibitory potency (18.03% and 20% for Ca$^{2+}$ and HPO$_4^{2-}$ ions respectively) as compared to 0.25 ml of more than 10 kDa fraction. In addition, the maximum inhibitory potency attained by 1.25 ml of crude aqueous extract is 82.14% for Ca$^{2+}$ and 80.04% for HPO$_4^{2-}$ ions, which is lower than the maximum inhibitory potency attained by more than 10 kDa fraction at the same volume. This clearly shows that percentage of inhibition by more than 10 kDa extract is much higher as compared to the crude extract of *Trachyspermum ammi* at same volume and same concentrations.

In contrast, the inhibitory potency of less than 10 kDa extract was found to be drastically less as compared to the inhibitory activity of crude extract. Figure 3.6 represents the inhibitory activity by different volumes of fractions having biomolecules of less than 10 kDa molecular weight. From the figure 3.6, it is clear that the inhibitory potential of less than 10 kDa fraction is glaringly less. The maximum percentage of inhibition shown by this fraction is 15.45% and 17.33% for Ca$^{2+}$ and HPO$_4^{2-}$ ions respectively at 0.75 ml of fraction. After 0.75 ml till 1.25 ml of fraction, the percentage of inhibition remained more or less same. The percentage inhibition by 1.25 ml of this extract is 12.34% and 13.4% for Ca$^{2+}$ and HPO$_4^{2-}$ ions respectively (figure 3.6 a & b).
**Results**

*In vitro antilithiatic activity of plants*

**Figure 3.5a.** Percentage inhibition of calcium ions by more than 10 kDa fraction of *Trachyspermum ammi*.

**Figure 3.5b.** Percentage inhibition of phosphate ions by more than 10 kDa fraction of *Trachyspermum ammi*.

**Figure 3.5.** Evaluation of more than 10 kDa fraction of 10% (w/v) *Trachyspermum ammi* aqueous extract on CaP initial mineral phase formation (Values are mean ± SD, n = 5).
Figure 3.6a. Percentage inhibition of calcium ions by less than 10 kDa fraction of *Trachyspermum ammi*.

Figure 3.6b. Percentage inhibition of phosphate ions by less than 10 kDa fraction of *Trachyspermum ammi*.

Figure 3.6. Evaluation of less than 10 kDa fraction of 10% (w/v) *Trachyspermum ammi* aqueous extract on CaP initial mineral phase formation (Values are mean ± SD, n = 5).
Similarly, the activity of both more than and less than 10 kDa was also evaluated against calcium oxalate crystal growth. The inhibitory activity of more than 10 kDa is shown in figure 3.7. It could be observed from the figure that more than 10 kDa fraction showed about 46% inhibition at 5 μl of sample and the activity increased gradually to 65.4% with 20 μl of sample.

In addition, it was also found that the more than 10 kDa extract have a higher inhibitory potency as compared to its crude aqueous extract. The percentage of inhibition by 5 μl of more than 10 kDa fraction was significantly higher (46.9%) as compared to the activity of 5 μl of crude aqueous extract (7.9%) as depicted in figure 3.4. The highest percentage of inhibition achieved by 20 μl of more than 10 kDa fraction was 65.4% whereas the highest percentage of inhibition by 20 μl of crude aqueous extract is 43.2%.

**Figure 3.7.** Evaluation of more than 10 kDa fraction of 10% (w/v) *Trachyspermum ammi* aqueous extract on CaOx crystal growth (Values are mean ± SD, n = 5)
Results

The inhibitory activity of less than 10 kDa extract on calcium oxalate crystal growth is shown in figure 3.8. The less than 10 kDa extract showed about 3.4% inhibition by 5 µl of sample. The percentage of inhibition increased considerably to 17.9% by 10 µl of the sample. Highest percentage of inhibition of CaOx crystal growth is shown by 20 µl of sample i.e. about 34.6%.

It could be inferred from the figure 3.7 and 3.8 that more than 10 kDa extract has a higher inhibitory potency (65.4% by 20 µl of sample) as compared to less than 10 kDa extract (34.6% by 20 µl of sample). In addition more than 10 kDa fraction showed much higher inhibitory potency towards CaOx crystal growth as compared to its crude extract whereas less than 10 kDa extract showed a much lower activity to inhibit CaOx crystal growth as compared to crude aqueous extract. This further ascertain that biomolecules of more than 10 kDa molecular weight are having higher inhibitory potency as compared to biomolecules having less than 10 kDa molecular weight. Thus, the phytochemical analysis was done to qualitatively identify the type of biomolecules possessing inhibitory potential towards both CaP and CaOx crystallization.

![Graph showing inhibition of calcium oxalate crystal growth](image)

**Figure 3.8.** Evaluation of less than 10 kDa fraction of 10% (w/v) *Trachyspermum ammi* aqueous extract on CaOx crystal growth (Values are mean ± SD, n = 5)
Both fractions having more than and less than 10 kDa extract were qualitatively screened for proteins and various phytochemicals viz. tannins, saponins, terpenoids, flavonoids and alkaloids by the method as described in section 2.4.5. The presence and absence of above mentioned biomolecules is depicted in table 3.1. The table clearly indicates that more than 10kDa fraction contains only proteins whereas less than 10kDa extract contains many other biomolecules like saponins, tannins, terpenoids, flavonoids and proteins. The molecular weight range of saponins, alkaloids, terpenoids and flavonoids is usually below 10 kDa, whereas tannins and proteins are such biomolecules whose molecular weight can be both less than and more than 10 kDa.

**Table 3.1.** Qualitative estimation of phytochemicals in the more than and less than 10 kDa fraction of *Trachyspermum ammi* aqueous extract.

<table>
<thead>
<tr>
<th>Phytochemicals</th>
<th>More than 10 kDa fraction</th>
<th>Less than 10 kDa fraction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tannins</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Saponins</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Alkaloids</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Terpenoids</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Flavonoids</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Proteins</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>
3.2. Identification and purification of antilithiatic protein

Exploratory studies using modern technologies to define novel CaOx crystal growth inhibitors are necessary and may lead to better understanding of the mode of action by which phytotherapy works to inhibit the formulation of kidney stones. From the previous results shown in section 3.1.5, it is clear that more than 10 kDa fraction of *Trachyspermum ammi* has higher ability to inhibit CaP mineralization whereas its less than 10 kDa fraction possess much lower potential to inhibit CaP mineralization. Since, more than 10 kDa extract has proteins as its major constituents, so a strategy to purify the most effective antilithiatic protein was adopted.

The major antilithiatic biomolecule from the seeds of *Trachyspermum ammi* was purified using chromatographic methods such as ammonium sulfate precipitation, anion exchange chromatography and molecular sieve chromatography. A graphic representation of the purification process adopted is given below in figure 3.9.

![Diagram]

**Figure 3.9.** Graphical representation of purification process adopted
3.2.1. Ammonium sulfate precipitation

Among the three step purification procedure, the first step was ammonium sulfate precipitation. The principle and method of ammonium sulfate precipitation is given in section 2.5.2. Protein was precipitated from the crude extract by addition of excess of ammonium sulfate salt at increasing saturation. The precipitate so obtained was tested for its potency to inhibit CaP mineralization and CaOx crystal growth (Table 3.2). From table 3.2, it is clear that maximum amount of protein got precipitated at 40-60% of ammonium sulfate saturation, which is about 194.91 mg. The amount of protein precipitated by 0-20% precipitation and non precipitate fraction above 80% precipitation was least among other fractions.

Table 3.2. Inhibitory potential of precipitates obtained after ammonium sulfate precipitation of Trachyspermum ammi crude extract.

<table>
<thead>
<tr>
<th>Precipitation range (%)</th>
<th>Total protein content (mg)</th>
<th>CaP inhibitory activity (1000μg/ml of Protein Sample)</th>
<th>CaOx inhibitory activity (1000μg/ml of Protein Sample)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0-20</td>
<td>15.07</td>
<td>9.40 ± 0.11 %age inhibition of Ca&lt;sup&gt;2+&lt;/sup&gt; ions</td>
<td>10.86 ± 0.49 %age inhibition of HPO&lt;sub&gt;4&lt;/sub&gt;&lt;sup&gt;2-&lt;/sup&gt; ions</td>
</tr>
<tr>
<td>20-40</td>
<td>25.17</td>
<td>20.91 ± 3.1 %age inhibition of Ca&lt;sup&gt;2+&lt;/sup&gt; ions</td>
<td>18.71 ± 3.4 %age inhibition of HPO&lt;sub&gt;4&lt;/sub&gt;&lt;sup&gt;2-&lt;/sup&gt; ions</td>
</tr>
<tr>
<td><strong>40-60</strong></td>
<td><strong>194.91</strong></td>
<td><strong>63.2 ± 2.1</strong> %age inhibition of Ca&lt;sup&gt;2+&lt;/sup&gt; ions</td>
<td><strong>60.91 ± 1.87</strong> %age inhibition of HPO&lt;sub&gt;4&lt;/sub&gt;&lt;sup&gt;2-&lt;/sup&gt; ions</td>
</tr>
<tr>
<td>60-80</td>
<td>67.18</td>
<td>47.72 ± 2.55 %age inhibition of Ca&lt;sup&gt;2+&lt;/sup&gt; ions</td>
<td>44.53 ± 2.92 %age inhibition of HPO&lt;sub&gt;4&lt;/sub&gt;&lt;sup&gt;2-&lt;/sup&gt; ions</td>
</tr>
<tr>
<td>&gt;80</td>
<td>19.79</td>
<td>16.44 ± 1.17 %age inhibition of Ca&lt;sup&gt;2+&lt;/sup&gt; ions</td>
<td>15.52 ± 1.14 %age inhibition of HPO&lt;sub&gt;4&lt;/sub&gt;&lt;sup&gt;2-&lt;/sup&gt; ions</td>
</tr>
</tbody>
</table>
Results

Identification and purification of antilithiatic protein

In addition, the inhibitory activity of protein content obtained at 60% saturation was highest as compared to the activity of other precipitates. The inhibition rate of CaOx crystal growth was 51% and inhibition of Ca$^{2+}$ and HPO$_4^{2-}$ ions by the protein obtained after 60% saturation was 63.2% and 60.91% respectively. The inhibitory activity of protein content precipitated at 60-80% saturation was moderate (21.39% for CaOx growth, 47.72% Ca$^{2+}$ and 44.53% for HPO$_4^{2-}$), which can also be linked with the moderate protein content in this fraction. Although none of the fractions showed a negative inhibition towards both assay systems, but the protein obtained by 40-60% saturation showed maximum inhibitory activity.

3.2.2. Anion exchange chromatography

The protein obtained after 40-60% saturation of ammonium sulfate precipitation was subjected to further purification on anion exchange chromatography. The protein fraction was concentrated and centrifuged to remove any non-dissolving materials and loaded over a Macro Prep 25 Q Strong anion exchanger (Bio-Rad laboratories) column to separate the proteins present in the sample on the basis of their charge.

Figure 3.10 shows the elution profile of the protein after anion exchange chromatography by LP Data view version 1.03 software. In this figure, the blue line indicates the absorbance unit (AU) of the sample eluted w.r.t. time, measured at wavelength 280 ($\lambda_{280}$). The red line indicates the conductivity of the sample eluted w.r.t. time, signifying the gradient of salt achieved during elution.

The eluting proteins were categorized into various peaks depending on the absorbance of solution, and are consecutively numbered as peak 1, 2, 3, 4, 5, 6, 7, 8 and 9. The solutions present in all tubes under each peak were pooled. The salt was removed from the each pooled peak and further the salt-free solution was lyophilized. The lyophilized protein was resuspended in buffer, such that the concentration of the protein remained 1mg per ml. After achieving the similar concentration of proteins under each peak, the inhibitory activity towards CaP and CaOx crystallization was studied.
Figure 3.10. Elution profile generated by LP data view after anion exchange chromatography
Table 3.3. Inhibitory potential of fractions obtained after anion exchange chromatography

<table>
<thead>
<tr>
<th>Peak</th>
<th>Pooled fractions (mints)</th>
<th>Total protein content</th>
<th>CaP inhibitory activity (1000μg/ml of Protein)</th>
<th>CaOx inhibitory activity (1000μg/ml of Protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>%age inhibition of Ca(^{2+}) ions</td>
<td>%age inhibition of HPO(_{4})^{2-} ions</td>
</tr>
<tr>
<td>1</td>
<td>10-17</td>
<td>47.27</td>
<td>20.91 ± 1.33</td>
<td>18.44 ± 2.7</td>
</tr>
<tr>
<td>2</td>
<td>18-21</td>
<td>18.81</td>
<td>9.3 ± 0.82</td>
<td>7.54 ± 1.12</td>
</tr>
<tr>
<td>3</td>
<td>22-27</td>
<td>12.14</td>
<td>2.23 ± 0.59</td>
<td>1.93 ± 0.36</td>
</tr>
<tr>
<td>4</td>
<td>28-35</td>
<td>9.21</td>
<td>17.42 ± 2.72</td>
<td>18.56 ± 2.91</td>
</tr>
<tr>
<td>5</td>
<td>36-42</td>
<td>37.41</td>
<td>69.12 ± 2.18</td>
<td>75.16 ± 1.51</td>
</tr>
<tr>
<td>6</td>
<td>43-47</td>
<td>11.13</td>
<td>15.17 ± 3.51</td>
<td>12.53 ± 2.44</td>
</tr>
<tr>
<td>7</td>
<td>48-54</td>
<td>3.12</td>
<td>negligible</td>
<td>negligible</td>
</tr>
<tr>
<td>8</td>
<td>56-67</td>
<td>39.14</td>
<td>32.85 ± 3.79</td>
<td>36.15 ± 2.38</td>
</tr>
<tr>
<td>9</td>
<td>68-75</td>
<td>7.26</td>
<td>11.63 ± 1.2</td>
<td>8.53 ± 25</td>
</tr>
</tbody>
</table>

Table 3.3 is showing the protein content and their corresponding inhibitory activity towards CaP and CaOx crystallization. The table shows all peaks, their corresponding time of elution, the amount of protein eluted and their respective inhibitory activity. Peak 1, eluted at time interval of 10-17 minutes, before the start of gradient (Figure 3.10) have the maximum content of protein (47.27 mg) but a moderate inhibitory activity towards both assay systems. The peak 5, eluted at time interval of 36 to 42 mins is showing the maximum percentage of inhibition towards CaP mineralization and CaOx crystal growth. The total amount of protein under this peak was 37.41 mg and the conductivity range of its elution is 27.43 mS/cm and 44.45 mS/cm.
Peak 4 and peak 8 are two other peaks which showed some inhibitory potency towards both CaP and CaOx crystallization, since peak 5 has maximum inhibitory potential and focus is to find the most potent antilithiatic protein, therefore peak 5 was subjected to SDS-PAGE to test its purity.

3.2.3. Determining the purity and composition of 5th peak by SDS-PAGE analysis

The fraction having maximum inhibitory potential eluted after anion exchange chromatography under peak 5 was further tested for its purity and composition using SDS PAGE analysis. A 10% gel was used for SDS-PAGE analysis. The protein of peak 5 was run parallel with protein molecular weight markers. Figure 3.11 is showing the band pattern obtained after SDS PAGE analysis of peak 5.

![SDS-PAGE Image](image.png)

**Figure 3.11.** Composition of peak 5 (36-42 mins) by SDS-PAGE (10%) analysis. Lane (1) is peak 5, Lane (2) is molecular weight markers
The first lane in figure 3.11 is of peak 5 and the second lane is showing molecular weight markers with their corresponding weights tagged with it. The SDS PAGE profile of peak 5 clearly showed that this elution is a mixture of about 5 proteins and this fraction needs further purification to isolate the most active antilithiatic protein. For the fractionation of various proteins under this peak molecular sieve chromatography was employed.

3.2.4 Molecular sieve chromatography

After isolation of proteins based on their charge, the proteins eluted under peak 5 were subjected to molecular sieve chromatography, which partitions the proteins on basis of their molecular weights. The proteins of peak 5 were lyophilized, concentrated and centrifuged to remove any non-dissolving material before loading over molecular sieve column. Figure 3.12 is showing the elution profile i.e. absorbance unit (AU) of the solution eluted w.r.t. time after molecular sieve chromatography. The eluting proteins were categorized into various peaks depending on the OD of solution, and are consecutively numbered as peak 1, 2, 3, 4 and 5, shown in figure 3.12.

The total protein content and their subsequent inhibitory activity towards both assay systems is given in table 3.4. The first peak eluted a very small amount of protein (0.67 mg) which has a negligible activity towards both assay systems. Other peaks followed by first, i.e. peak 2, 3, 4 and 5, showed some extent of inhibition towards both CaP and CaOx crystallization.

Although the maximum amount of protein is eluted under peak 3 i.e. 5.92 mg, but still peak 4 (3.84 mg of protein) is having maximum ability to inhibit CaP and CaOx crystallization. Peak 4 eluted between the range of 980 to 1072 minutes, showed the highest inhibitory potential towards CaP mineralization (77.15% for Ca$^{2+}$ and 75.32% for HPO$_4^{2-}$ ions) and CaOx crystal growth (83.01%).
Figure 3.12. Elution profile generated by LP data view after molecular sieve chromatography
Table 3.4. Inhibitory potential of fractions obtained after molecular sieve chromatography

<table>
<thead>
<tr>
<th>Peak</th>
<th>Pooled fractions (mins)</th>
<th>Total protein content</th>
<th>CaP inhibitory activity (1000μg/ml Sample) % age inhibition of Ca^{2+} ions</th>
<th>% age inhibition of HPO_{4}^{2-} ions</th>
<th>CaOx inhibitory activity (1000μg/ml of Protein Sample)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>63-146</td>
<td>0.67</td>
<td>negligible</td>
<td>negligible</td>
<td>negligible</td>
</tr>
<tr>
<td>2</td>
<td>214-307</td>
<td>1.98</td>
<td>41.85 ± 3.83</td>
<td>38.69 ± 1.73</td>
<td>30.73 ± 1.15</td>
</tr>
<tr>
<td>3</td>
<td>625-951</td>
<td>5.92</td>
<td>23.64 ± 3.11</td>
<td>21.28 ± 2.19</td>
<td>17.36 ± 0.74</td>
</tr>
<tr>
<td>4</td>
<td>980-1072</td>
<td>3.84</td>
<td>77.15 ± 1.71</td>
<td>75.32 ± 2.03</td>
<td>83.01 ± 3.06</td>
</tr>
<tr>
<td>5</td>
<td>1084-1218</td>
<td>4.12</td>
<td>16.23 ± 2.82</td>
<td>16.84 ± 1.54</td>
<td>21.58 ± 2.4</td>
</tr>
</tbody>
</table>

Followed by peak 4, peak 2 and 3 also showed moderate inhibitory activity towards CaP and CaOx crystallization. Extensive profiling of all these fractions with inhibitory activity against CaP and CaOx crystal growth and their characterization might throw light on current knowledge of other modulators of stone formation present in *Trachyspermum ammi*.

The most effective antilithiatic fraction i.e peak 4 was further tested for its purity by SDS PAGE analysis.

3.2.3. Determining the purity and composition of 4th peak after molecular sieve chromatography by SDS-PAGE analysis

The purity of 4th peak obtained after molecular sieve chromatography was tested by SDS PAGE analysis and is shown in figure 3.13.
Figure 3.13 shows the protein profile of most active fraction and it could be found that the fraction eluted between the time period of 980 to 1072 minutes, showed a single band by SDS-PAGE analysis. The first lane (Lane 1) is showing the SDS-PAGE profile of peak 4 and second lane (Lane 2) consists of molecular weight markers.

From the figure 3.13, on comparing the band position of peak 4 with molecular weight markers, it could be interpreted that the protein band of peak 4 has a molecular weight of more than 100 kDa.

![Image of SDS-PAGE gel showing molecular weight markers and protein bands]

**Figure 3.13.** Composition of peak 4 (980-1072 mins) by SDS-PAGE (10%) analysis. Lane (1) is peak 4, Lane (2) is molecular weight markers
3.2.6. Outline of purification procedure adopted

The three step purification procedure adopted to isolate an antilithiatic protein is concisely given in table 3.5. The buffer extract from 60 gm of *Trachyspermum ammi*’s powdered seeds, yielded 321.92 mg of protein having the inhibitory activity of 48% towards CaOx crystal growth and for CaP it showed 55.39% and 53.73% for Ca$^{2+}$ and HPO$_4^{-2}$ ions respectively.

As the purification procedure progressed, the activity of most potent fraction was found to increase subsequently. The yield in percentage is also mentioned in the table and it can be found that after molecular sieve chromatography there was an abrupt decrease in the yield. The final yield of the active protein was found to be 1.1%.

**Table 3.5. Outline of purification of inhibitory protein from the seeds of *Trachyspermum ammi*. %age inhibition of CaOx represents results as mean ± SD (n = 6). Data refer to the protein obtained during various stages of purification from 60gms of powdered *Trachyspermum ammi* seeds.**

<table>
<thead>
<tr>
<th>Purification Steps</th>
<th>Total Protein (mg)</th>
<th>Yield (%)</th>
<th>CaP inhibitory activity (1000μg/ml of Protein)</th>
<th>% Inhibition of CaOx by 1000μg/ml of protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>Buffer Extract</td>
<td>321.92</td>
<td>100</td>
<td>55.39 ± 3.21</td>
<td>48 ± 1.47</td>
</tr>
<tr>
<td>60% (NH$_4$)$_2$SO$_4$ precipitation</td>
<td>194.91</td>
<td>60.5</td>
<td>63.2 ± 2.1</td>
<td>51 ± 1.09</td>
</tr>
<tr>
<td>Anion exchange chromatography</td>
<td>37.41</td>
<td>11.6</td>
<td>69.12 ± 2.18</td>
<td>79 ± 1.62</td>
</tr>
<tr>
<td>Molecular sieve chromatography</td>
<td>3.84</td>
<td>1.1</td>
<td>77.15 ± 1.71</td>
<td>83 ± 3.06</td>
</tr>
</tbody>
</table>
3.3. Characterization of purified antilithiatic protein

The knowledge of proteins that can inhibit stone formation is limited to a relatively small number of proteins. Identification of additional stone-inhibitory proteins was hampered in the past by limitations in protein identification methods. An exploratory study using modern technologies to define and characterize novel calcium oxalate crystal growth inhibitors is necessary. So, the antilithiatic protein isolated and purified from the seeds of *Trachyspermum ammi* was further characterized to understand the possible mechanism by which this protein inhibits CaP and CaOx crystallization.

3.3.1. Homogeneity of purified protein by HPLC

As shown in figure 3.12 the fraction having maximum inhibitory potential towards both CaP mineralization and CaOx crystal growth, eluted at the 980 min to 1072 min, presented a single band in SDS-PAGE gel. Although this single band, showed that this fraction has a single protein, but a more precise confirmation was done by RP-HPLC. A single peak was observed after elution of the protein loaded over RP (C-18) column on HPLC. The retention time of the protein on C-18 column was 12.04 min (Figure 3.14). Thus, the active fraction was confirmed for having a single antilithiatic protein and this protein will be referred to as *Trachyspermum ammi* antilithiatic protein (TAP) in the text of this thesis.

3.3.2. *Trachyspermum ammi* antilithiatic protein (TAP) molecular mass determination

After the confirmation of homogeneity of the active fraction the molecular mass of the protein was determined. Figure 3.13 shows SDS-PAGE analysis of the purified protein which revealed single band of molecular mass more than 100 kDa (approximate). The exact molecular mass of purified protein was determined by size exclusion HPLC using Protein Pak 125 column. The log of molecular mass of standard proteins viz carbonic anhydrase 29kDa; bovine serum albumin 68kDa; alcohol dehydrogenase 150kDa, was calculated to be 4.46, 4.83 and 5.17 respectively.
Figure 3.14. Homogeneity ascertained by a single peak on RP-HPLC

Figure 3.15. Molecular weight determination of TAP by size exclusion HPLC
Results

Characterization of purified antilithiatic protein

On plotting the retention time of these standard proteins with respect to their log of molecular mass, a straight line was procured as shown in fig 3.15. The retention time of the *Trachyspermum ammi* antilithiatic protein was 16.8. On extrapolating this retention time on y-axis of the graph, the molecular mass of the purified protein was calculated to be 107 kDa (Figure 3.15) on a standard curve plotted using protein markers in the range of molecular mass 29-150 kDa.

3.3.3. Dose dependent response of purified protein towards calcium oxalate and calcium phosphate assay

The effect of varying concentration of TAP was studied on CaP and CaOx assay systems, to know the dose dependent response of purified protein. Figure 3.16 shows the percentage of CaOx crystal growth inhibitory activity after 5 minutes of incubation of *Trachyspermum ammi* antilithiatic protein (TAP) at 50 µg/ml, 100 µg/ml, 200 µg/ml, 350 µg/ml and 450 µg/ml. It is evident from the figure 3.16 that with increase in concentration of protein from 50 µg/ml to 100 µg/ml, its inhibitory activity also increased. But as the concentration of this protein increased further from 100 µg/ml to 200 µg/ml, 350 µg/ml and 450 µg/ml, the inhibitory activity towards CaOx crystal growth remained almost unchanged.

The evaluation of inhibitory potency of isolated protein (TAP) on calcium phosphate crystallization, using similar concentrations of protein is shown in figure 3.17. On comparing the percentage inhibition of both calcium and phosphate ions after incubation with TAP at increasing concentrations, a similar trend as calcium oxalate crystal growth inhibition was observed. A drastic increase in percentage of inhibition of both Ca$^{2+}$ and HPO$_4^{2-}$ ions on increasing the concentration of TAP from 50 µg/ml to 100 µg/ml was observed, but increase in concentration of TAP after 100 µg/ml showed either no increase or just a marginal increase in the inhibitory activity towards both Ca$^{2+}$ and HPO$_4^{2-}$ ions.
Figure 3.16. Dose dependent effect of TAP on calcium oxalate crystal growth

Figure 3.17. Dose dependent effect of TAP on calcium phosphate mineralization
3.3.4. Total amino acid composition of TAP

To find the composition of total amino acid present in DAP, a HPLC based method formulated by Elkin & Wasyczuk [219] was employed. The acid hydrolysis of DAP resulted in disintegration of the protein into free amino acids. These free amino acids on analysis by HPLC using silica based column (Pico-tag), gave peaks corresponding to their elution time. The peaks were identified using elution time with standards of every amino acid and the area under each peak gave the corresponding concentration of that amino acid in the acid hydrolyzate of TAP protein.

The HPLC chromatogram showed peaks, and each peak represents elution of a particular amino acid. The amino acids and their corresponding elution time were depicted. There was no peak in the HPLC chromatogram corresponding to amino acid tryptophan; this is because tryptophan is completely destroyed after acid hydrolysis of protein. Additionally, the glutamine and asparagine are converted into glutamic acid and aspartic acid respectively, so they appear as a single peak after elution.

Table 3.6 shows the percentage of each amino acid in TAP. It was found that the percentage of acidic amino acids like aspartic acid and glutamic acids were present in maximum amount in TAP protein. There percentage composition is 9.4% and 12.18% for both aspartic acid and glutamic acid respectively. Followed by acidic amino acids, basic amino acids viz. lysine and arginine were also found to be present in good amount (7.4% and 7.9% respectively).

From these results, it was also found that in TAP protein, polar amino acids serine was also present in adequate amount and it constituted about 9.19% of the total amino acids. The aliphatic amino acids and other aromatic amino acids were also present in TAP but in less amount. The content of amino acids viz. alanine, leucine, isoleucine and valine were 6.53 %, 8.31 %, 5.4% and 3.09% respectively in the TAP. The aromatic amino acids like phenylalanine and tyrosine are present in fewer amounts i.e. 1.61% and 4.31% respectively.
Table 3.6. Percentage of amino acids in TAP

<table>
<thead>
<tr>
<th>S.No</th>
<th>Amino Acids</th>
<th>Amino acid percentage in TAP</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Alanine</td>
<td>6.53</td>
</tr>
<tr>
<td>2</td>
<td>Arginine</td>
<td>7.94</td>
</tr>
<tr>
<td>3</td>
<td>Aspartic acid*</td>
<td>9.41</td>
</tr>
<tr>
<td>4</td>
<td>Cysteine</td>
<td>0.65</td>
</tr>
<tr>
<td>5</td>
<td>Glutamic acid**</td>
<td>12.18</td>
</tr>
<tr>
<td>6</td>
<td>Glycine</td>
<td>8.35</td>
</tr>
<tr>
<td>7</td>
<td>Histidine</td>
<td>3.47</td>
</tr>
<tr>
<td>8</td>
<td>Isoleucine</td>
<td>5.42</td>
</tr>
<tr>
<td>9</td>
<td>Leucine</td>
<td>8.32</td>
</tr>
<tr>
<td>10</td>
<td>Lysine</td>
<td>7.40</td>
</tr>
<tr>
<td>11</td>
<td>Methionine</td>
<td>5.78</td>
</tr>
<tr>
<td>12</td>
<td>Phenylalanine</td>
<td>1.69</td>
</tr>
<tr>
<td>13</td>
<td>Proline</td>
<td>2.72</td>
</tr>
<tr>
<td>14</td>
<td>Serine</td>
<td>9.19</td>
</tr>
<tr>
<td>15</td>
<td>Threonine</td>
<td>3.60</td>
</tr>
<tr>
<td>16</td>
<td>Tryptophan</td>
<td>-</td>
</tr>
<tr>
<td>17</td>
<td>Tyrosine</td>
<td>4.31</td>
</tr>
<tr>
<td>18</td>
<td>Valine</td>
<td>3.10</td>
</tr>
</tbody>
</table>

*include both aspartic acid and asparagines
**include both glutamic acid and glutamine
3.3.5. Isoelectric point determination

The isoelectric point of the purified protein was evaluated from the figure 3.18. From the figure slope (m) and Y-intercept (b) of straight line was calculated to be 0.75 and -4.219 respectively.

The value of higher plateau and lower plateau were measured from figure 3.15 and it was found to be .Putting these values in the formula, the isoelectric point was calculated as follows.

\[
\text{pI} = \frac{1}{0.75} \left( \frac{0.021 + 0.88}{2} + 4.219 \right) = 6.2
\]

Isoelectric point of protein was found to be 6.2.

**Figure 3.18.** Determination of Isoelectric point of TAP and found to be 6.2
3.3.6. Spectroscopic measurements

The light absorption character of the protein was studied in UV and visible region to find out its spectroscopic properties. Figure 3.19 shows the spectrum of protein TAP. The protein showed peak at $\lambda_{280}$ nm as shown in figure. There was no absorbance observed in the visible region of the spectrum. In additionally the protein was colorless even at very high concentration indicating absence of heme group.

![Spectroscopic analysis of TAP](image)

**Figure 3.19.** Spectroscopic analysis of TAP between wavelength ($\lambda$) range of 220-400nm. The $\lambda_{\text{max}}$ was found to be 280nm

3.3.7. Peptide mass fingerprinting by MALDI-TOF MS

The mixture of peptides obtained after trypsinization of TAP was loaded on a MALDI TOF mass spectrometer. The MALDI-TOF MS gave the m/z ratio of all peptides in the digested TAP sample. Figure 3.20 shows the peptide mass fingerprinting, the graph is between m/z ratios of peptides versus the intensity of the peptide. MALDI-TOF MS segregate the peptides on the basis of their mass to charge ratio. As a result each peptide moves with different speed in the analyzer and depending on their time of flight, the peptides reach at different times to the detector. Thus, with MALDI-TOF MS we can detect the number of peptides and their m/z ratios. In figure 3.20, various peaks are shown with their corresponding m/z ratios.
Figure 3.20. The peptide mass fingerprinting by MALDI-TOF MS obtained from trypsinized TAP
3.3.8. Protein Matching

The m/z ratio of all peptides as observed by MALDI TOF MS, were loaded in mascot search engine. Mascot search engine compares the m/z ratios acquired by MALDI TOF MS with all proteins’ in the data base, if they were digested with trypsin. Figure 3.21 presents the results obtained after Mascot search. The m/z ratios of peptides of TAP after searching with mascot search engine showed the maximum similarity with unnamed protein product of *Vitis vinifera* (CAO23876). The match showed sequence coverage of 44% of TAP and the peptides which are showing an exact match are marked red in figure 3.21.

**Mascot Search Results**

Protein View

Match to: gi|157329022 Score: 69 Expect: 0.034
*unnamed protein product [Vitis vinifera]*

Nominal mass (M): 87461; Calculated pI value: 6.13
NCBI BLAST search of gi|157329022 against nr
Unformatted sequence string for pasting into other applications

Taxonomy: *Vitis vinifera*

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: 95
Number of mass values matched: 40
Sequence Coverage: 44%

Matched peptides shown in Bold Red

1. MASRASLQKR RYLPNSLNNP TCCVROGFSSF EHGASSQFNE SRGRFIMATS
51. PQSNDTPPRK GYFLSLSKKE LSSSFLSLGLL RHNIGGISTL GGCRRRTDFI
101. SLPEQGCVVSQ YIHVYSLTIA QPQLDQGQIN ENEEQNAKPK KEASPEECOQ
151. AVEGLSTYKM ERKAKQCQES QROGKVYVIRK QQHMILGIGG GAPAVASMHR
201. EDWAALKQSH KDEDHSMDH YMGLTLSLMA DVRLRRLLK LLAGGKLSLR
251. RERQQLTRTT ADFILVPVFA VFIIIIPFNEF LPLFLHLFP NLNPSTFQDK
301. MKEQELARKK LNRLEKAIY IQDTKEKEMAK EVQUNSHGEGI KTAEDLDOQF
351. HVRRTGAGV SNDEILQFAR LHDETELTDH IRSPRLVNH CDMGISPYGT
401. DALIYRLHRL RLQYNKDDVR MIQAEQVESL SEARELQACR DRLLGAPSF
451. GFRLQQLPKLL SLLFITLSTYA FPSISRSOGD LTYLSILONW PEACSGKLVH
501. LEKELPLVV KVRLDDLAL ELKCOVEQGL PRLQILPLLA PFKSVYVEEEE
551. KEEVQVARK ESTVQKQDKVA LEENTITPAR EAQEQAEAKT LEKQQQICEL
601. SRALVLASL SSQSWFREAF LRLVRKEIYL YHHEVEKERT EDEEFAEAY
651. RSARGKSDRA VHEAVADAKS SALIDVDRDAH LOKLEKIDD VDAKIGDRWR
701. LLKRDYDGVK TPEVMSATH YLRDLGSLGD IQEELSHLSK DKEKIRFV
751. IIMLGERDR DSSDPRGV

**Figure 3.21.** Results of MASCOT search engine after loading peptide m/z ratio from TAP
3.3.9. Putative function of protein and domain identification

The sequence of unnamed protein product of *Vitis vinifera* (UPVV) was obtained from NCBI protein database and to check its family, the sequence was BLAST with non-redundant database. Figure 3.22 is showing the results after BLAST.

<table>
<thead>
<tr>
<th>Sequences producing significant alignments:</th>
<th>Score (Bits)</th>
<th>E Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>emb:CA023876.1</td>
<td>unrelated protein product [Vitis vinifera]</td>
<td>1581</td>
</tr>
<tr>
<td>emb:CAN97346.1</td>
<td>hypothetical protein [Vitis vinifera]</td>
<td>828</td>
</tr>
<tr>
<td>ref:NP_001047387.1</td>
<td>Os02g0660800 [Oryza sativa (japonica cultivar)]</td>
<td>682</td>
</tr>
<tr>
<td>gb:AA66304.1</td>
<td>Ca2+ binding protein cbp1 [Triticum aestivum]</td>
<td>814</td>
</tr>
<tr>
<td>gb:CA236626.1</td>
<td>hypothetical protein Os1_007361 [Oryza sativa ...</td>
<td>813</td>
</tr>
<tr>
<td>gb:CA237654.1</td>
<td>hypothetical protein Os1_007246 [Oryza sativa ...</td>
<td>813</td>
</tr>
<tr>
<td>ref:NP_181541.1</td>
<td>calcium-binding mitochondrial protein-related...</td>
<td>779</td>
</tr>
<tr>
<td>emb:CAH57493.1</td>
<td>hypothetical protein [Oryza sativa (indica cultivar-grown)]</td>
<td>771</td>
</tr>
<tr>
<td>ref:NP_001033262.1</td>
<td>Os04g0496000 [Oryza sativa (japonica cultivar)]</td>
<td>771</td>
</tr>
<tr>
<td>ref:NP_001030897.1</td>
<td>calcium-binding mitochondrial protein-related...</td>
<td>757</td>
</tr>
<tr>
<td>gb:CA294704.1</td>
<td>hypothetical protein Os1_015937 [Oryza sativa ...</td>
<td>723</td>
</tr>
<tr>
<td>ref:NP_001770225.1</td>
<td>predicted protein [Physcomitrella patens ...</td>
<td>669</td>
</tr>
<tr>
<td>emb:CA046335.1</td>
<td>hypothetical protein [Vitis vinifera]</td>
<td>647</td>
</tr>
<tr>
<td>emb:CAN97347.1</td>
<td>hypothetical protein [Vitis vinifera]</td>
<td>559</td>
</tr>
<tr>
<td>ref:NP_187672.2</td>
<td>calcium-binding EF hand family protein [Arab...</td>
<td>556</td>
</tr>
<tr>
<td>gb:AA663090.1</td>
<td>Similar to Saccharomyces hypothetical protein ...</td>
<td>537</td>
</tr>
<tr>
<td>ref:XP_001419090.1</td>
<td>predicted protein [Ostreococcus lucimarin...</td>
<td>351</td>
</tr>
<tr>
<td>ref:XP_001779222.1</td>
<td>predicted protein [Physcomitrella patens ...</td>
<td>340</td>
</tr>
<tr>
<td>emb:CA458441.1</td>
<td>Ca2+-binding transmembrane protein LETM1/MRS?</td>
<td>277</td>
</tr>
<tr>
<td>gb:AAV43090.1</td>
<td>unknown [Zea mays] &gt;gb:AAV43090.1</td>
<td>275</td>
</tr>
<tr>
<td>gb:AA663097.1</td>
<td>EST gb:NB7484 comes from this gene. [Arabidopsis]</td>
<td>259</td>
</tr>
<tr>
<td>ref:NP_0010006461.1</td>
<td>leucine zipper-EF-hand containing transmembrane</td>
<td>257</td>
</tr>
<tr>
<td>ref:XP_171216.1</td>
<td>hypothetical protein UN04169.1 [Ustilago may...</td>
<td>256</td>
</tr>
<tr>
<td>ref:XP_002146728.1</td>
<td>hypothetical protein TRIADRAFT_60795 [Tr...</td>
<td>255</td>
</tr>
<tr>
<td>ref:XP_001605311.1</td>
<td>predicted protein [Nematostella vectensis]</td>
<td>253</td>
</tr>
<tr>
<td>ref:NP_001388673.1</td>
<td>hypothetical protein LOC570745 [Danio rer...</td>
<td>252</td>
</tr>
<tr>
<td>ref:XP_001374738.1</td>
<td>predicted protein [Monodera sp. ...</td>
<td>247</td>
</tr>
<tr>
<td>ref:NP_001069082.1</td>
<td>leucine zipper-EF-hand containing transmembrane</td>
<td>244</td>
</tr>
<tr>
<td>ref:NP_001072793.1</td>
<td>leucine zipper-EF-hand containing transmembrane</td>
<td>243</td>
</tr>
<tr>
<td>emb:CA057391.1</td>
<td>hypothetical protein [Tetradon nigroviridis]</td>
<td>242</td>
</tr>
<tr>
<td>ref:XP_966965.1</td>
<td>PREDICTED: similar to paramyxovirus, putative ...</td>
<td>236</td>
</tr>
<tr>
<td>ref:XP_001400448.1</td>
<td>predicted protein [Aelurocytes capsa...</td>
<td>234</td>
</tr>
<tr>
<td>ref:XP_001450543.1</td>
<td>paramyxovirus [Culex quinquefasciatus ...</td>
<td>233</td>
</tr>
<tr>
<td>ref:XP_001818378.1</td>
<td>hypothetical protein [Aspergillus oryzae ...</td>
<td>233</td>
</tr>
</tbody>
</table>

Figure 3.22. BLAST of unnamed protein product of *Vitis vinifera* CAO23876 with non-redundant database
Results

Characterization of purified antilithic protein

The BLAST showed that protein belongs to LETM1 superfamily. It was also found that sequence of this protein had similarity with NP_191541 (calcium-binding mitochondrial protein-related, Arabidopsis thaliana); AAW66005 (Ca2+ binding protein cbp1, Triticum aestivum); NP_176732 (calcium-binding EF hand family protein, Arabidopsis thaliana) thus indicating that the protein belong to calcium binding EF hand protein family. Figure 3.23 shows domains identified in unnamed protein of *Vitis vinifera* (CAO23876) by SMART normal module. This protein has a LETM1 domain (207-461) and two EF hand domain (694-722 and 730–757). The sequence of two EF hand domains in the protein are also shown in figure 3.23.

![Diagram showing domains](image)

**EF Hand (694-722):** KIGDRWRLLDRDYDGKVTPEEVASATMYL

**EF Hand (730-757):** GIQELISNLSKDEGKIRVEDIIKLGSE

**Figure 3.23.** Domains identified in unnamed protein of *Vitis vinifera* (CAO23876) by SMART normal module. The position of LETM1 domain (207-461) and two EF hand domain (694-722 and 730 – 757) are marked. The sequence of both EF hand domains is also represented

3.3.10. Interaction of active binding domain with calcium oxalate crystals

The two EF hand domains in the protein were considered as active domains for imparting antilithic activity, so these domains were further studied *in vitro* to check their interaction with calcium oxalate crystal (most predominant in kidney stones).
The structure of calcium oxalate monohydrate crystal was obtained from Cambridge structure database (www.ccdc.cam.ac.uk). The structure of COM is shown in figure 3.24 as observed by software Mercury crystal structure (MCS). The green color bond is between calcium and oxygen (Ca-O), red bond is between two oxygen atoms, grey color bond is present in oxalate group, between carbon and oxygen and white color bond is depicting the hydrate moiety i.e. the bond between hydrogen and oxygen in water molecule. The figure 3.24 also represents free calcium binding sites for further growth which will be denoted as growth points or the site of further growth of COM.

**Figure 3.24.** The structure of Calcium oxalate monohydrate (COM) unit cell showing coordination polyhedra of atoms Calcium 1 [Ca (1)] and Calcium 2 [Ca (2)].

The energy of the structure of COM was minimized by using force field MM2 with dielectric constant equal to 1.2 to obtain the most stable structure of COM. This minimized (energy) structure of COM was treated as a ligand for further docking simulations.
The structure of active binding site of two EF hand domains identified in CAO23876 protein shown in figure 3.23, were modeled using MOE. After adding the hydrogen atoms and prior to the docking calculations, an energy minimization using MMFF94 forcefield was performed on both binding sites using the minimization protocol of Steepest Descent (SD), Conjugate Gradient (CG) and Truncated Newton (TN) methods. During the minimization, non-hydrogen atoms were held fixed and the RMSD gradient used in SD was 1,000, in CG was 100, and in the TN step the RMSD was 0.1. The iteration limits in SD and CG are 100 in TN 200.

3.3.10.1. Docking simulations

Both the EF hand domains were docked with COM crystal and table 3.7 represents docking score and the free energy of binding of both EF hand domains on interaction with COM as well as the amino acid sequence of binding sites.

Table 3.7. The docking score and estimated free energy of binding ($\Delta G_{binding}$) on interaction of EF hand domain with the unit cell of COM

<table>
<thead>
<tr>
<th>Active binding domain</th>
<th>Sequence</th>
<th>Docking score (kcal/mol)</th>
<th>Free binding energy (kcal/mol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 EF hand a</td>
<td>KIGDRWLLLDRDYDGKVTP \newline EEVASATMYL</td>
<td>516.260</td>
<td>-12.00</td>
</tr>
<tr>
<td>2 EF hand b</td>
<td>GIQELISNLSDKDEGKIRVED \newline IIKLGSE</td>
<td>22.530</td>
<td>-16.09</td>
</tr>
</tbody>
</table>

After docking both active binding domains (EF hand a and EF hand b) with COM crystal, a negative score of free binding energy was observed. This presents that both the domains interacted efficiently with COM crystal. Moreover, it was found that the interaction of EF hand b (-16.09) was stronger as compared to EF hand a (-12.00). Both the docked structures were analyzed by LIGPLOT analysis to check the involvement of amino acids of the domain causing these strong interactions.
### 3.3.10.2. LIGPLOT analysis

The docked structure of COM and both EF hand domains were further analyzed by LIGPLOT analysis. Table 3.8 shows the amino acids of these active binding sites which are actively involved in interaction with COM. The ionic bonding and hydrophobic interactions between the amino acids and the atoms of COM crystal and their corresponding bond distance is depicted in the table 3.8.

**Table 3.8.** Ionic bonds and hydrophobic interaction between the EF hand domains and COM crystal

<table>
<thead>
<tr>
<th>Ionic bonding</th>
<th>Hydrophobic interaction</th>
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<tr>
<td>Ionic species 1</td>
<td>Ionic species 2</td>
</tr>
<tr>
<td>EF hand a (KIGDRWRLLDRDYDGKVTPEEVASATMYL)</td>
<td></td>
</tr>
<tr>
<td>42 CA</td>
<td>GLU A 714 OE</td>
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<tr>
<td>EF hand b (GIQELISNLSDKKEGKIRVEDIIGLGSE)</td>
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<tr>
<td>52 CA</td>
<td>VAL A 748 O</td>
</tr>
<tr>
<td>1 CA</td>
<td>GLU A 733 O</td>
</tr>
<tr>
<td>1 CA</td>
<td>GLU A 733 OE</td>
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Figure 3.25. Two dimensional representations of the interactions observed between COM unit cell and EF hand a domain. Dashed lines denote ionic bond (or hydrogen bonds), and numbers indicate bond lengths in Å. Hydrophobic interactions are shown as arcs with radial spokes. The figure was made using LIGPLOT.
**Key**

- **Ligand bond**
- **Non-ligand bond**
- **Hydrogen bond and its length**
- **Non-ligand residues involved in hydrophobic contact(s)**
- **Corresponding atoms involved in hydrophobic contact(s)**

**Figure 3.26.** Two dimensional representations of the interactions observed between COM unit cell and EF hand b domain. Dashed lines denote ionic bond (or hydrogen bonds), and numbers indicate bond lengths in Å. Hydrophobic interactions are shown as arcs with radial spokes. The figure was made using LIGPLOT.
The 2-dimensional image depicting hydrogen bonding and hydrophobic interactions between amino acids with COM crystal is shown figure 3.25 and 3.26. Figure 3.25 shows the interaction of EF hand a domain with COM crystal. From the figure it can be seen that a strong bond is formed between $O^\alpha$ of glutamic acid at position 714 with calcium atom at position 46 i.e the growing site of COM crystal. Probably this interaction was involved in inhibiting further growth of COM crystal.

In addition to ionic bonding, certain hydrophobic interactions were also found to be involved in causing strong interactions of COM with this domain. Glutamic acid at 714 position is also involved in hydrophobic interaction with carbon atom of oxalate ion. Other amino acids implicated in hydrophobic interactions are Ala at position 718 and Trp at position 699. Although the EF hand a domain has one more Glu at position 713 but this amino acid showed no bonding of either ionic bond or hydrophobic interactions with COM unit cell.

Figure 3.26 is showing the two dimensional representation of EF hand b interaction with COM crystal. In this domain Glu at position 733 was found to form two strong bonds with calcium atom at position 1 of COM crystal. The first bond is formed between $O$ of Glu and calcium atom and the second bond is formed between $O^\alpha$ of Glu with same calcium atom. The strongest bond (2.3 Å) among two is first one.

In the EF hand b domain Val was also found to be involved in forming strong bond with COM crystal. Here O of Val formed ionic bond with calcium atom at position 52 of COM crystal. The aliphatic amino acids like Gly and basic amino acid like Lys was found to be involved in forming hydrophobic interactions. Gly at position 744 formed hydrophobic interactions with carbon at position 64 and 24 and Lys at position 754 was found to form hydrophobic interactions with carbon at position 26.
3.4. In vivo experimentation on hyperoxaluric rats

To elucidate the in vivo efficacy of *Trachyspermum ammi* seeds, a hyperoxaluric rat model was used as described in section 2.8. The evaluation of antilithiatic property was ascertained by administrating purified antilithiatic protein of *Trachyspermum ammi* to hyperoxaluric rats. During the treatment period, all rats in TAP treated groups reacted normally to both doses (i.e. 1 mg and 2 mg per kg body weight). But in the groups exposed to EG and NH₄Cl for 9 and 15 days, there was a loss of one rat in each, at the end of treatment period. After 4th day of the treatment period, the rats started showing lethargic behavior and got confined to the corners of cages.

After the treatment period, the required samples were collected from the rats and various biochemical and histological parameters were estimated. The results of all treatment are given as follows.

3.4.1. Body weight measurement

The rats were weighed before and after the treatment period to see the effect of all treatments on them. Figure 3.27 is showing the body weight of rats in all groups after the completion of treatment period. Figure 3.27a is showing the change in body weight of rats after treatment period of 9 days. After 9 days of exposure to EG and NH₄Cl, a highly significant decrease ($p>0.001$) in the body weight of about 25% in comparison to control group 1 was observed. It was found that after the completion of treatment period of 9 days, the body weight of rats in group P3 (1 mg per kg body weight) and P4 (2 mg per kg body weight) was significantly ($p>0.01$) high as compared to hyperoxaluric group P2 rats.

The body weight of rats given treatment for 15 days is shown in figure 3.27b. In the rats of group Q2, given EG and NH₄Cl for 15 days, showed a marked deterioration in their body weight. The decrease in body weight of these rats was about 30% as compared to control group Q1 rats. Here again the rats in group Q3 and Q4, given TAP dose intraperitoneally in addition to EG + NH₄Cl and showed a meager decrease in their body weights.
Results

In vivo experimentation on hyperoxaluric rats

Figure 3.27a. Effect of all treatments on body weight of rats after 9 days

Figure 3.27b. Effect of all treatments on body weight of rats after 15 days

Figure 3.27. Influence of both treatment periods on body weight of rats after both 9 and 15 days

[*p < 0.05, **p < 0.01, ***p < 0.001]: Indicate significant change in comparison to control group P1/Q1.

[p < 0.05, ###p < 0.001]: Indicate significant change between group P2/Q2 and P3/Q3. *p < 0.05.

##p < 0.01, ^^^p < 0.001: Indicate significant change between group P2/Q2 and P4/Q4. Values in brackets are % increase or % decrease as compared to group P1/Q1].
Results

In vivo experimentation on hyperoxaluric rats

**Figure 3.28a.** Urine volume excreted in 24 hrs by rats under treatment period of 9 days

![Graph showing urine volume for Group P1 to P4 with percentage changes indicated](image)

**Figure 3.28b.** Urine volume excreted in 24 hrs by rats under treatment period of 15 days

![Graph showing urine volume for Group Q1 to Q4 with percentage changes indicated](image)

**Figure 3.28.** Urine volume excreted in 24 hrs under treatment period of 9 and 15 days

[*p < 0.05, **p < 0.01, ***p < 0.001: Indicate significant change in comparison to control group P1/Q1. #p < 0.05, ##p < 0.01, ###p < 0.001: Indicate significant change between group P2/Q2 and P3/Q3. ^p < 0.05, ^^p < 0.01, ^^^p < 0.001: Indicate significant change between group P2/Q2 and P4/Q4. Values in brackets are % increase or % decrease as compared to group P1/Q1.]
Results

In vivo experimentation on hyperoxaluric rats

**Figure 3.29a.** Activity of urinary alkaline phosphatase after 9 days treatment

**Figure 3.29b.** Activity of urinary alkaline phosphatase after 15 days treatment

**Figure 3.29.** Influence on the activity of urinary alkaline phosphatase after both 9 and 15 days treatment

[^p<0.05, ^*p<0.01, ^**p<0.001: Indicate significant change in comparison to control group P1/Q1. #p<0.05, ##p<0.01, ###p<0.001: Indicate significant change between group P2/Q2 and P3/Q3. ^*p<0.05, ^^^p<0.01, ^^^^p<0.001: Indicate significant change between group P2/Q2 and P4/Q4. Values in brackets are % increase or % decrease as compared to group P1/Q1.**
3.4.2. Urine analysis

The urine from all rats was collected in metabolic cages as described in section 2.8.3. Before acidifying the urine and storing it for further analysis, its volume was measured. Figure 3.28a shows the 24 hrs volume of urine procured on 8th day of treatment. It is clearly shown in figure 3.28a and 3.28b that group P2 and Q2 rats which were given EG and NH₄Cl for 9 and 15 days respectively, showed a higher amount of urine excretory volume as compared to control group rats. The percentage increase in the urine volume of rats treated for 9 days was 50% and rats treated for 15 days was 60% respectively. The rats treated with TAP also showed a significantly ($p<0.001$) higher content of urine when compared to control group 1 rats. On comparing the urinary volume of group P2 with P3 & P4 and Q2 with Q3 & Q4, it was found that there was no significant difference in the urine content and the urine was higher in these groups as compared to control rats.

3.4.2.1. Urinary alkaline phosphatase

The alkaline phosphatase activity in the urine was measured as a urine injury marker enzyme. Figure 3.29 is showing the activity of alkaline phosphatase enzyme in the urine sample of rats in all groups. The activity of alkaline phosphatase, in group P2 rats exposed to EG and NH₄Cl for 9 days was significantly ($p<0.001$) higher as compared to control rats (Figure 3.29a). The percentage rise in its activity in these rats was about 112%, representing double amount of AP activity in these rats as compared to control group P1. In contrast, the activity of AP in group P3 and P4 which were given TAP injection in addition to exposure of EG + NH₄Cl, although showed a rise in AP activity (60% for group P3 and 23% for P4) w.r.t P1 control but the activity of AP was significantly ($p>0.05, p>0.01$) lower than AP activity of group P2 rats (Figure 3.29a).

Similarly, in group Q2 given EG and NH₄Cl for 15 days showed a marked increase in activity of urinary AP. Group Q2 rats showed a rise of 137% in AP activity as compared to control group Q1 rats. Here again, group Q3 and Q4 given 1 mg per kg body
weight and 2 mg per kg body weight of TAP respectively showed a significant ($p>0.001$ and $p>0.01$) decrease in AP activity as compared to group Q2 rats.

On comparing the activity of AP in urine of group P3 and P4 with group Q3 and Q4, it could be said that the percentage decrease in AP activity in comparison to EG and NH$_4$Cl exposed groups, after 15 days of TAP administration was more than that after 9 days administration of TAP. Therefore, EG + NH$_4$Cl exposure after 15 days to group Q2 was more aggravating as compared to its 9 days exposure. The administration of TAP for 15 days in group Q3 and Q4 was more curative in reducing AP activity as compared to group P3 and P4, in which rats were administered TAP for 9 days.

3.4.2.2. Urinary lactate dehydrogenase activity

The activity of urinary lactate dehydrogenase after both 9 and 15 days treatment period in all the groups is shown in figure 3.30. The LDH activity in group P2 which were given EG and NH$_4$Cl dose for 9 days showed a marked increase in LDH activity in these animals. The significant ($p>0.001$) increase in LDH activity in these group rats was about 65% as compared to control group P1 rats. The P3 group animals were given 1 mg per kg body weight of TAP in addition to ethylene glycol, although the animals showed an increase in LDH activity but still the rise in its level was 24% as compared to control group P1 and it was significantly lower than group P2 animals. Furthermore, the animals given higher dose of TAP (2 mg/kg body weight) again presented a significant decrease in LDH activity as compared to group P2. Additionally, the rats in group P4 showed a marginal rise in LDH activity as compared to control group P1. The treatment period of 15 days also showed a similar type of trend. Here the activity of urinary LDH in group Q2 was much higher (73%) as compared to group P2 (65%). Similar to 9 days treatment period, the rats in group Q3 and Q4 showed a significant ($p>0.001$ and $p>0.01$ respectively) decrease in LDH activity as compared to group Q2. Although the LDH activity in group Q3 and Q4 rats was higher than control group Q1 rats, but still percentage increase in LDH activity in these rats was just about 24% and 12% respectively for group Q3 and Q4 when compared to control group Q1 rats. On comparing the level of activity of LDH in TAP treated rats after 9 days and 15 days treatment, it was found that restoration of LDH activity in terms of percentage change from control was same.
Results

In vivo experimentation on hyperoxaluric rats

**Figure 3.30a.** Activity of urinary lactate dehydrogenase after 9 days treatment

**Figure 3.30b.** Activity of urinary lactate dehydrogenase after 9 days treatment

**Figure 3.30.** Influence on the activity of urinary lactate dehydrogenase after 9 and 15 days treatment

[*p < 0.05, **p < 0.01, ***p < 0.001: Indicate significant change in comparison to control group P1/Q1. 
#p < 0.05, ##p < 0.01, ###p < 0.001: Indicate significant change between group P2/Q2 and P3/Q3. *p < 0.05, 
^^p < 0.01, +++p < 0.001: Indicate significant change between group P2/Q2 and P4/Q4. Values in brackets are % increase or % decrease as compared to group P1/Q1].
3.4.2.3. **Urinary polarization microscopy**

Figure 3.31 shows the polarization light micrographs of bladder urine of rats after 9 days of treatment period. These micrographs revealed that urine of group P1 rats was more or less devoid of any crystal whereas in group P2 rats, the bladder urine sample showed presence of abundant bipyramidal shaped COD and dumbbell-shaped COM crystals. The aggregates of these crystals were also visible at many instances as shown in figure 3.30b.

In the urine of group P3 and P4 rats, a drastic decrease in the number of urinary crystals was observed. Urine polarization micrograph of group P3 rats exhibited presence of only few COD and a negligible number of COM crystals as shown in figure 3.31c. Similarly, group P4 rats given higher dose of TAP, illustrates the presence of only COD crystals and here again there number was quite less as shown in figure 3.31d. Additionally, the sizes of COD crystals in P4 group rats were very small in comparison to COD crystals of group 2 rats.

Likewise, group Q1 was also devoid of any crystal deposition but the animals of group Q2 given ethylene glycol and NH₄Cl for 15 days showed abundant COM and COD crystals as shown in figure 3.32b. The TAP treated urolithiatic animals of group Q3 and Q4 showed very few aggregates of COD or COM crystals (figure 3.32c & 3.32d). Though the urine of group Q3 animals revealed no crystal aggregates, but still their urine presented a higher number of COM and fewer COD crystals (Figure 3.32c).

Correspondingly, urolithiatic animals treated with a higher dose of TAP at 2 mg/kg body weight (group Q4) showed fewer crystals in the urine and here again COM crystals were pre-dominant. In the figure 3.32d, it is also shown that at many instances the crystals observed were broken and disintegrated. The size of COM crystals in these group animals was also very small as compared to urolithiatic group B2 animals.
Results

In vivo experimentation on hyperoxaluric rats

Figure 3.31a.

Figure 3.31b.
Figure 3.31. Polarization micrographs of rat’s urine after 9 days treatment
Figure 3.32a.

Figure 3.32b.
Figure 3.32. Polarization micrographs of rat’s urine after 15 days treatment
3.4.3. Serum analysis

After collection of serum, the serum level of urea and creatinine was estimated as shown below.

3.4.3.1. Serum urea content

Figure 3.33 shows the serum urea content and the level of significance among all groups. The content of serum urea after 9 days of EG + NH₄Cl treatment, showed that there is a drastic increase in the content of urea in serum and this ascend is significant ($p>0.001$) when compared with serum urea content in control rats. The content of serum urea in hyperoxaluric group P2 rats was almost double the content of serum urea of control animals. On administering TAP at a dose of 1 mg per kg body weight to hyperoxaluric rats in group P3, the serum urea content was still higher as compared to control group P1, but its content was significantly ($p>0.01$) lower when compared to hyperoxaluric rats of group P2. The rats administered TAP at a higher dose of 2 mg per kg body weight similarly presented a significant ($p>0.01$) decrease in serum urea content in group P4 rats as compared to hyperoxaluric rats of group P2.

The consequences of treatment period of 15 days are shown in figure 3.33b. From the figure it can be clearly seen that yet again the rise in serum urea content in hyperoxaluric group Q2 rats was much higher as compared to its subsequent control group Q1. The serum urea content in this group was more than double (115%) the amount of serum urea of control group Q1. The treatment of group Q3 and Q4 rats with TAP resulted in decrease in amount of serum urea as compared to group Q2. The higher dose of TAP (2 mg per kg body weight) showed a better restoration of urea content to control levels.

The dose of EG + NH₄Cl for 15 days presented a higher increase in serum urea content as compared to same dose for 9 days. In addition, the restoration of serum urea level by TAP after 15 days administration was more near to control level as compared to its 9 days treatment.
Results

In vivo experimentation on hyperoxaluric rats

**Figure 3.33a.** Content of serum urea after 9 days treatment

**Figure 3.33b.** Content of serum urea after 15 days treatment

Figure 3.33. Influence on the content of serum urea after 9 and 15 days treatment

\[^{*}p<0.05, \text{ ***}p<0.001\]: \text{Indicate significant change in comparison to control group P1/Q1.} \\
\[^{#}p<0.05, \text{ ###}p<0.001\]: \text{Indicate significant change between group P2/Q2 and P3/Q3.} \\
\[^{**}p<0.05, \text{ ####}p<0.001\]: \text{Indicate significant change between group P2/Q2 and P4/Q4.} \text{Values in brackets are \% increase or \% decrease as compared to group P1/Q1.}
Results

In vivo experimentation on hyperoxaluric rats

Figure 3.34a. Content of serum creatinine after 9 days of treatment

Figure 3.34b. Content of serum creatinine after 15 days of treatment

Figure 3.34. Influence on the content of serum creatinine after 9 and 15 days of treatment

[*p < 0.05, **p < 0.01, ***p < 0.001: Indicate significant change in comparison to control group P1/Q1. 
#p < 0.05, ##p < 0.01, ###p < 0.001: Indicate significant change between group P2/Q2 and P3/Q3. *p < 0.05, 
^^p < 0.01, ^^p < 0.001: Indicate significant change between group P2/Q2 and P4/Q4. Values in brackets are % increase or % decrease as compared to group P1/Q1]
**Results**

*In vivo experimentation on hyperoxaluric rats*

**Figure 3.35a.** Level of creatinine clearance in all groups after 9 days treatment

**Figure 3.35b.** Level of creatinine clearance in all groups after 9 days treatment

**Figure 3.35.** Influence on level of creatinine clearance after 9 and 15 days treatment

[*p < 0.05, **p < 0.01, ***p < 0.001: Indicate significant change in comparison to control group P1/Q1. #p < 0.05, ##p < 0.01, ###p < 0.001: Indicate significant change between group P2/Q2 and P3/Q3. *p < 0.05, ^^p < 0.01, ^^^p < 0.001: Indicate significant change between group P2/O2 and P4/O4. Values in brackets are % increase or % decrease as compared to group P1/Q1.*]
3.4.3.2. Serum creatinine

Figure 3.34 shows the content of serum creatinine after 9 and 15 days treatment in all the groups of rats. Figure 3.34a is depicting the content of serum creatinine in all four groups given treatment for 9 days. From the figure, it can be clearly noticed that the exposure of group P2 rats to EG and NH₄Cl, causing hyperoxaluric conditions, had resulted in a significant ($p>0.001$) increase of serum creatinine content by 52% as compared to control group P1.

In contrary, the rats of group P3 exposed to hyperoxaluric conditions in addition to administration of TAP, showed a significant ($p>0.01$) decrease in creatinine content as compared to hyperoxaluric rats of group P2. Though, a higher dose of TAP (7 mg/kg body weight) to group P4 depicted a significant ($p>0.001$) increase in serum creatinine content, yet it was significantly ($p>0.01$) less as compared to hyperoxaluric group P2. In addition, the increase in serum creatinine content of group P3 was about 25% and in group P4 it was about 17% in comparison to group P1.

On estimating the serum creatinine content in hyperoxaluric group Q2 after similar treatment of 15 days, an increase of about 59% in creatinine content was observed as compared to control group Q1. Similar to the trend observed for the treatment period of 9 days, the treatment of hyperoxaluric rats with TAP for 15 days showed a reduction in creatinine content in the serum as compared to group Q2. In addition, the decrease in creatinine content after TAP administration was dose-dependant.

3.4.4. Creatinine clearance

The creatinine clearance (CrCl) in all groups after both treatment periods is represented in figure 3.35. The hyperoxaluric rats (group P1) given EG and NH₄Cl portrayed a highly significant ($p>0.001$) decrease of about 72% in CrCl. The rats of this group presented about half of creatinine being cleared off per unit time as compared to control group P1. The TAP administration for 9 days showed restitution of CrCl in both groups P3 and P4. The increase in CrCl content in these group rats is highly significant ($p>0.001$) as compared to hyperoxaluric rats of group P2.
The CrCl in all groups after treatment period of 15 days is represented in figure 3.35b. From the figure, it could be easily interpreted that after 15 days of exposure of rats to hyperoxaluric dose resulted in a marked (75%) decrease in CrCl by these group rats. The level of CrCl in these group rats was 1.14 ml/min. Administration of TAP to group Q3 and Q4 showed a significant (p>0.001) increase in CrCl in comparison to hyperoxaluric group Q2.

From figure 3.35b it could be concluded that the dose of EG and NH₄Cl for 15 days caused more deleterious effect of CrCl as compared to 9 days dose. Additionally, the treatment of hyperoxaluric rats for 15 days with TAP (group Q3 and Q4) showed better restoration of CrCl (w.r.t. hyperoxaluric group Q2) for both doses as compared to 9 days administration (w.r.t. hyperoxaluric group P2). The effect of TAP on CrCl was also a dose-dependant response.

3.4.5. Histological analysis of kidney tissue

The transverse section of kidney tissue was fixed and stained with hemotoxylin & eosin and viewed under light and polarization microscope. The features of kidney tissue revealed by both microscopy are depicted below.

3.4.5.1. Kidney tissue under light microscope

Kidney tissue after H & E staining is viewed under light microscope. The light micrographs of kidney tissue are shown in figure 3.36. The figure 3.36a, illustrates the normal morphology of kidney tissues. The histology of control rats presented normal globular glomerulus within bowman’s capsule. The bowman’s capsule was intact and lined with a continuous lining of epithelial cells. The renal tubules showed intact lumen lined by cuboidal epithelial cells. The histology of control group P1 rats showed no sign of inflammation and hemolysis.

On the other hand, the kidney histology of hyperoxaluric rats (as shown in figure 3.36b) given ethylene glycol and NH₄Cl showed a highly distorted morphology. The glomerulus has lost its globular characteristic and it appeared shrunken. The epithelial
lining of Bowman’s capsule was found to be broken at many instances. The renal tubules had lost their intact structure as no distinct lumen was observed and the cuboidal epithelial of renal tubules was disintegrated. In addition to these morphological alterations, the histology of rats in this group showed marked inflammation and hemolysis. The cells of these rats were distorted and signs of edema were also observed at many instances in their histology.

The rats in group P3 which were treated with TAP at a dose of 1 mg per kg body weight portrayed a restored morphology as compared to control group P1 rats (Figure 3.36c). In the histology of these rats, the signs of inflammation were reduced and no hemolysis and edema was observed. Though the globular form of glomerulus was not restored completely, the rats of this group showed normal size of glomerulus. Among other signs of alteration, the loss of intact renal tubules was still prevalent at many instances even after treatment with TAP.

Correspondingly, in figure 3.36d, the histology of rats given TAP at the dose of 2 mg/kg body weight is shown. From the figure, it could be clearly seen that the histology of these animals is comparable with control animals. The glomerulus is of appropriate size and show globular nature to some extent. Glomerulus of these animals was lined by intact Bowman’s capsule. There were many instances of intact renal tubules lined by cuboidal epithelial cells in the histology of these rats. The decrease in tissue injury was also evidently observed. The renal histology of group P4 rats was more near to control rats than group P3 thus indicating that TAP showed a dose-dependant response towards restoration of kidney histology.

Figure 3.37 is showing the kidney histology of rats under light microscope, given treatment period for 15 days. The rats in control group Q1 showed normal morphology similar to the morphology of rats in group P1 as shown in figure 3.37a. The histology of renal tissue after 15 days treatment, presented that medullary rays were intact and arranged properly in control rats and the tubules of medullary rays are lined by continuous cuboidal epithelial.
Results

In vivo experimentation on hyperoxaluric rats

Alternatively, the rats given dose of ethylene glycol and ammonium chloride for 15 days (group Q2) showed obvious changes in their histology. The kidney tissue of these rats showed marked hemolysis at many instances. The kidney tissues of these rats presented a noticeable cell rupture as is shown in figure 3.37b. The arrangement of epithelial cells in their tissue was completely lost. The signs and instances of inflammation and hemolysis in these rats were significantly more than in the rats of group P2 given same dose for 9 days.

The rats given TAP dose at 1 mg/kg body weight for 15 days to group Q3 demonstrated some marks of renal histology restoration. In group Q3, the occurrence of hemolysis and inflammation in the kidney tissue was less as compared to group Q2. From the histological analysis of group Q3 rats, it can be suggested that although the arrangement of epithelial cells in kidney tissue is not completely restored, but the rupturing of epithelial cells in their tissue is evidently less as compared to hyperoxaluric group Q2 rats kidney tissue (Figure 3.37c ).

The kidney histology of group Q4 rats given TAP at a higher dose of 2 mg/kg body weight, showed much improved histology as compared to hyperoxaluric group Q2. The epithelial cell rupturing was completely missing in these rats kidney tissues. The epithelial cells lining the renal tubules showed improved arrangement and there was a marked decrease in inflammation of kidney tissue of these rats. In addition, the kidney tissue of group Q4 rats showed almost negligible signs of hemolysis as compared to hyperoxaluric group Q2.

The dose-dependant response of TAP was evidently observed in kidney histology restoration. The rats of group Q4 showed better restitution of epithelial cells and decreased inflammation in the kidney tissue as compared to group Q3.
Results

In vivo experimentation on hyperoxaluric rats

Figure 3.36a

Figure 3.36b
**Figure 3.36.** Kidney tissue observed under light microscope (9 days treatment)

[Magnification: X200]
Results

In vivo experimentation on hyperoxaluric rats

Figure 3.37a

Figure 3.37b
Figure 3.37c

Figure 3.37d

**Figure 3.37.** Kidney tissue observed under light microscope (15 days treatment)  
[Magnification: X200]
3.4.5.2. Polarization microscopy of kidney tissue

The slides of kidney tissues stained with H & E were also viewed under polarization microscope. The polarization microscopy focuses the crystals deposited in the kidney tissue. Figure 3.38a is showing the polarization micrographs of control group P1 rats. It was evidently found that the kidney tissues of this group of rats showed no mark of crystal deposition.

The histology of kidney tissue of rats given ethylene glycol and NH₄Cl for 9 days is shown in figure 3.38b. In this figure, it is evidently clear that the rats of group P2, showed abundant crystal deposition in the tissue. The kidney tissue of all the rats in the rats in this group presented a huge number of crystal deposition and most of the crystals are deposited in the renal tubules. In addition to crystal depositions in renal tubules, the tubules also showed distorted morphology. There was no crystal deposition observed in the glomerulus. Since, renal tubules having crystal deposition is distorted, this clearly indicates that either crystal deposition has caused renal tubule distortion/injury or the injury in the renal tubule has lead to crystal accumulation in these tubules.

Figure 3.38c shows the renal tissue of group P3 under polarization microscope. In group P3 which were given TAP at 1 mg/Kg body weight, showed a marked decrease in number of crystal depositions in the kidney tissue. The crystals were observed at a few instances in renal tubules. The overall morphology of renal tissue was found to improve as compared to hyperoxaluric group P2 animals. The renal tubules were intact at many instances expect for those places where the crystal deposition was present.

Similarly, the TAP at a higher dose of 2 mg/Kg body weight in P4 group animals showed again a decrease in number of crystals (figure 3.38d). Here, again the animals showed the deposition of crystals in renal tubules and as in group A3, the animals in this group also showed decrease in renal injury. The reduced size as well as number of crystals and decreased renal injury again showed the efficacy of this protein in a dose-dependent manner.
Results

In vivo experimentation on hyperoxaluric rats

Figure 3.38a

Figure 3.38b
Figure 3.38. Polarization micrographs of renal tissue. [Magnification 200X]