To my Mom and Dad
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Kidney stone patients are often given aqueous extracts of the *Ammi visnaga* (Khella) plant in many middle and near eastern countries. The mode of action of Khella as a kidney stone therapy is not well understood. We postulated that components of the extract may inhibit crystallization of calcium oxalate (CaOx), the major component of most kidney stones, and prevent crystal retention within the kidneys. This study was carried out to learn the composition of the Khella extract and investigate the effect the extract has on crystallization of CaOx *in vitro*.

The composition of the plant extract was determined by Thin Layer Chromatography (TLC) and High Performance Liquid Chromatography (HPLC). Concentration of the free ions in the extract was determined by oxalate assay kits and inductive couple plasma (ICP). Crystal induction time in supersaturated CaOx solutions was determined at 37 °C using UV-VIS spectrometry. The induction time was estimated from the time vs. absorbance curve. Using an equation that relates induction times and supersaturation ratios, the surface energy, nucleation rate, free energy barrier, and critical nuclei radius were calculated. The interaction between free calcium and oxalate ions was determined by calcium titration. Aromic force microscopy (AFM) was used to study crystal-cell interaction between calcium oxalate monohydrate (COM) crystals.
and Marvin Darby Canine Kidney (MDCK) cells. The COM crystal was used in AFM measurements because the monohydrate structure is the most harmful to kidney epithelial cells.

Khella was obtained from two sources, one in Turkey and one in Egypt. The HPLC and TLC results showed that only Turkish Khella extract contained khellin and visnagin which are believed to be the active components of the herb. The results from ICP and oxalate determination kits showed that both extracts contained calcium, magnesium, and oxalate. The plant extract reduced the induction time at every supersaturation ratio. From the induction time data, free energy barrier and critical nuclei radius were estimated. The calculation revealed a decrease of free energy barrier and critical nuclei radius as supersaturation ratio increased. From the calcium titration experiments, it was determined that the addition of Khella extract maintained the amount of free calcium ions in the solution. Scanning electron microscopy (SEM) images showed that the control supersaturated CaOx solutions produced CaOx monohydrate (COM) crystals. With the addition of Khella, the resulting crystals were of the CaOx dihydrate (COD) form. The slope of the light absorbance measurement curve indicated the inhibition of calcium oxalate nucleation from Khella extract. AFM measurements showed no negative interaction force between COM crystal and MDCK cells therefore, the addition of Khella extract reduced the chance of COM crystals adhere to kidney epithelial cells.

The effect of individual components of the Khella extract, calcium, magnesium, khellin and visnagin, on calcium oxalate crystallization was also studied. All individual components identified, khellin, visnagin, calcium, and magnesium had no significant effect on kidney stone prevention in comparison to the extract. Therefore, we believe there are some unidentified components or a combination of components in Khella extract that are involved in the inhibition of kidney stone formation.
Khella extract caused an increase in crystallization induction time, a change in the type of calcium oxalate crystal produced (COM to COD), inhibition effects for both nucleation and aggregation of calcium oxalate crystals, and a non-adhesive interaction to kidney epithelial cells. COM crystals are considered more injurious than COD crystals. The efficacy of Khella extract as a therapy for stone disease may be a result of its effect on the crystallization of CaOx.
CHAPTER 1
INTRODUCTION

Kidney stone disease, also known as Nephrolithiasis and renal stones, is a common problem all over the world. It affects around 10% of the world’s population. The treatment of this disease costs over two billions dollar per year [1-3]. Kidney stone formation begins when crystals of calcium oxalate, the main component of kidney stones, are retained or deposited inside the kidneys, the crystals can grow or aggregate. The retained crystals block the urine flow leading to increased pressure inside the kidney. This results in pain and discomfort.

Less invasive methods beside surgery have been developed to treat kidney stone disease. One such method is extracorporeal shock wave lithotripsy (ESWL). ESWL uses shock waves to break kidney stones into small pieces that can easily pass through the urinary track[4]. Although, ESWL has been successful for treatment of kidney stone disease, many problems remain[1, 5]. Patients who undergo ESWL treatment may have increased incidences of hypertension and long-term renal damage. Stone fragments cannot be totally excreted from the kidney and may serve as sites for further nucleation and aggregation of crystals. ESWL is also an expensive procedure for patients.

Several chemical substances such as citrate, magnesium, and phosphate, taken orally, have been used to reduce the recurrence of kidney stones. However, these substances can cause some gastrointestinal side effects[6-9]. Therefore, an emphasis has been placed on the use of herbal medicinal treatments that are more cost efficient, are more effective, and have less side effects[10].

Herbal remedies, or folk medicines, are used in several parts of the world. In Egypt, Khella plant (*Ammi visnaga*) extract, used in the form of tea, has been found to stop the
reoccurrence of the kidney stones in the renal track[10, 11]. The plant extract has also been reported to help relax the ureter muscle, which reduces pain caused by the stones, and allows the stones to pass into the bladder more easily. However, the exact mechanism of Khella extract’s active components on stone crystallization has not yet been clearly identified. The organic and inorganic components of the Khella extract are suspected to play a role in inhibiting the nucleation and aggregation of calcium oxalate crystals. These components are believed to affect either the calcium or oxalate ion concentration in the urine and therefore the calcium oxalate nucleation rate. Also, they might absorb onto the crystals surfaces and change the calcium oxalate crystal solubility.

Kidney stones are composed of two phases, inorganic crystals and an organic matrix. The diuretic mechanisms of Khella extract’s active components on kidney stone formation could effect both phases of the stone matrix. This project investigates the effect of Khella extract on the crystallization of calcium oxalate, a main inorganic component of kidney stones, in the presence and absence of other urinary species such as citrate and proteins.
CHAPTER 2
LITERATURE SURVEY

Kidney Stone Disease

Kidney stone disease, nephrolithiasis or renal stone disease, is the most common urological disorder[1]. It is part of human biomineralization and refers to crystal deposition inside the kidneys. In the year 2000, approximately 2.7 million patients had problems related to kidney stones and around 177,496 had admitted to hospitals in United States[3, 12]. The economic impact on healthcare related to kidney stone treatment was estimated to be $2.07 billion in 2000, in United States alone[12]. Around 80-90 percent of people who form kidney stones will have a reoccurrence of the disease[1].

Kidney stones are formed from the ionic salt species found in urine. The most common biomineral forms of kidney stones are calcium oxalate, calcium phosphate, and uric acid. In most cases, the crystals formed are very small and can pass through the kidney and urological system harmlessly. However, in some cases crystals aggregate inside the kidney causing the blockage of urine flow out of the kidney resulting in increased pressure, pain, and infection. Depending on the size and location, untreated kidney stones can cause permanent damage to the kidneys[5].

Urinary Tract and Kidney Structure

The human urinary tract consists of the kidneys, ureters, bladder, and urethra (Figure 2-1). The kidneys work as a waste management center in the human body. They remove waste liquids, potentially harmful end products of metabolism, and extra water from the blood and convert them into urine. They also stabilize the essential substances in the blood such as water, sugars, amino acids, and electrolytes, as well as help produce hormones for building bones and generating red blood cells [3, 13]. The small tubes, called ureters, carry the urinary waste from
the kidneys to the bladder where it is collected. The body excretes urine from the bladder through the urethra.

![Diagram of the human urinary tract](image)

Figure 2-1 Structure of the human urinary tract [5].

The kidney consists of two major regions, the medulla and cortex (Figure 2-2). The medulla is the inner section and is divided into small coned-shape sections called renal pyramids. Each renal pyramid contains a papilla. Urine from renal papillae passes into renal pelvis and from there into ureter.

Each kidney in a human body contains roughly a million nephrons. The nephrons start in cortex act as small filtration units. They consist of a glomerular capillary network surrounded by a tubular section called Bowman’s capsule, a proximal tubule, a loop of Henle, a distal tubule, and a collecting duct. Each segment of nephron or renal tubules has a different ultra structure (Figure 2-3).
The waste products and excess water in the blood are forced past the glomerulus junction by capillary force. Then reabsorption of the essential salts, minerals and excess water begins throughout the tubular network. Each tubule is lined with a single layer of different types of epithelial cells on the top of a basal membrane. The tubular basal membrane provides the structural support for the epithelial cells and forms an uninterrupted framework throughout the length of the nephron. Various segments play significant roles in the formation of kidney stones[15].

The proximal convoluted tubule is where filtration starts and has a wider diameter than that of other sections involved in the reabsorption process. The epithelium structure of the proximal segment has a brush boarder area with compact, long and thin microvilli extensions. This structure provides high surface area to efficiently reabsorb important ionic salts back to the body.
The collecting duct is the last section of the nephron tubular system. End products and urine pass through the papillae, renal pelvis and finally the ureter.

Figure 2-3 Ultrastructural differences of the major epithelial cells in nephron. Schematic showing closed view of epithelial cell structure of a) proximal convoluted tubule and b) collecting duct[13].

The epithelial cells of the collecting duct segment are different from those in the proximal area. The cell brush boarder surface is composed of short microvilli that are less abundant and coarser than in the proximal segment (Figure 2-3b). This structure provides a smooth surface to
collect urinary waste but does not aid in reabsorption. Also, the length of microvilli structure changes the internal diameter of renal tubules. Therefore, the internal diameter of the proximal convoluted tubules is smaller because of the longer microvilli, while the collecting duct has larger internal diameter because of shorter microvilli. These differences in the microvilli structure on the epithelial cell surfaces may influence calcium oxalate crystal retention in the kidney and may be related to the formation of kidney stones. Therefore, two cell lines that resemble these two sections of the urinary tract have been used in most kidney stone related research[15-20]. The details of crystal-cell interaction will be discussed later on in the crystallization of calcium oxalate section.

### Composition and Structure of Kidney Stones

Kidney stones are composed of organic and inorganic components [21, 22]. Approximately 98 wt% of a kidney stone is the crystalline inorganic content, 90% of which is made of calcium oxalate mixed with calcium phosphate. Calcium oxalate crystals in the monohydrate form are the most typically found. There exists three hydrate forms of calcium oxalate; calcium oxalate monohydrate, calcium oxalate dihydrate, and calcium oxalate trihydrate. The morphologies of these crystal structures are shown in Figure 2-4. Calcium oxalate monohydrate (COM) is the most commonly found in stones because it is the most thermodynamically stable. Calcium oxalate dihydrate (COD), the metastable form, is also found and calcium oxalate trihydrate (COT) is rarely found in kidney stones. COT is believed to act as the precursor of the COD and COM crystals[23].

The difference in structure of each hydrate form can affect the renal epithelial cell condition. COM has sharp edges that can physically scrape the renal epithelial cells and result in cell injury while the other two hydrate structures have rounder and smoother edges. In addition,
the COM structure has a large oxalate-rich face which has been found to be harmful to the renal epithelial cells[16, 20]. Therefore, COM is the structure of focus in this study.

Figure 2-4 Calcium oxalate crystal in three hydrate forms; a) calcium oxalate monohydrate (COM), b) calcium oxalate dihydrate (COD), and c) calcium oxalate trihydrate (COT)[24, 25].

The organic matrix of the stone (around 2.5 %wt) consists primarily of small proteins with a molecular weight of 30,000 to 40,000 Da which may also include some larger molecules such as albumin, shredded epithelial cell membranes and their lipids, and other urinary proteins[13, 26]. The presence of the organic matrix in the stones suggests that homogenous nucleation of the stones is unlikely[15, 27]. Heterogeneous nucleation is more probable since urine is a very complex solution of water, ions, inorganic salts, small molecules, and macromolecules. The presence of these organic substances in urine can act as sites for heterogeneous nucleation[27]. Some urinary macromolecules such as the Tamm-Horsfall protein (THP), albumin and bikunin are also believed to play some role in stone crystallization[28-34]. However, there is no certainty
which protein(s) inhibits or promotes stone formation. The effects of macromolecules on stone crystallization depend on the ambient conditions \textit{in vivo}. Some proteins might have dual effects in crystallization by acting as promoter or inhibitor depending on the urinary environment[30, 32, 33, 35].

**Urine Composition and Its Role in Kidney Stone Formation**

Human urine is a naturally supersaturated solution of calcium oxalate. Therefore, it is normal for calcium oxalate crystals to nucleate in the urinary tract. As mentioned in the previous section, beside calcium and oxalate, urine is also composed of other inorganic salt ions, small molecules such as citrate and phosphate, macromolecules such as urinary proteins and lipids, and various metabolic wastes such as urea[36-38]. The urine composition can be related to the formation of kidney stones, as these components are present in both the organic matrix and inorganic crystalloids of the kidney stones[27]. Therefore, the interaction between urinary species affects stone formation. Most stone forming patients were found to have an abnormal level of calcium and oxalate in the urine, which results in higher tendency in forming stone[26, 39, 40].

Higher from normal calcium concentration in urine is referred to as hypercalciuria. However, hypercalciuria may be difficult to identify since the excretion of calcium also depends on a person’s diet and tolerable levels can naturally vary between patients. The excessive ingestion of calcium, e.g. drinking a lot of milk, or sodium may effect the excretion of calcium in the urine[26]. A high oxalate concentration in urine is referred as hyperoxaluria. The majority of kidney stone patients are diagnosed with an abnormal oxalate metabolism[13]. Not only does hyperoxaluria increase nucleation of crystals, but, a high oxalate concentration is associated with cell membrane injury [27]. When cells are exposed to a high concentration of oxalate either by the oxalate ion itself or as calcium oxalate crystals, the renal epithelia cells are injured and brush
border membrane is shredded and sloughed into the urine. This cell debris can act as a site for heterogeneous nucleation. The injured cells also expose the basement membrane which could provide a binding site for COM crystals.

**Crystallization of Calcium Oxalate in Biological Systems**

Crystallization of calcium oxalate needs to be investigated to achieve a better understanding of the stone formation mechanisms. Several researchers have suggested that some urinary species such as citrate or proteins such as THP act as inhibitors of kidney stone formation. More details on those studies will be discussed later in this dissertation. The crystallization process can be categorized into three main topics: nucleation, growth, and aggregation/dispersion.

**Nucleation Theory**

Nucleation is the first step of crystallization and supersaturation of the solution is a main driving force. Supersaturation occurs when the concentration of ionic species in solution exceed the saturation point. The supersaturation ratio (S) is calculated by[40]:

$$ S = \frac{c}{c^*} $$

(2-1)

where c and c* is solute concentration and solute solubility at the given temperature.

For nucleation to occur, the system has to overcome the energy barrier, Gibbs free energy of formation. The free energy of formation ($\Delta G_f$) is given by the sum of two contributions, the change in surface energy and the change in volume energy according to the following[37, 41]:

$$ \Delta G_f = 4\pi r^2 \gamma + \frac{4}{3} \pi r^3 \Delta G_v $$

(2-2)

where $\gamma$ is the surface free energy, in units of energy per unit area, $r$ is the radius of a solid cluster, and $\Delta G_v$ is the volume free energy. The plot of the free energy of formation as a function of radius is given in Figure 2-5:
From the schematic in Figure 2-5, the free energy of formation rises to a maximum as the radius of a solid cluster, \( r \), reaches the critical radius, \( r^* \), and then falls. This model suggests that before the solid clusters reach the critical radius size, they form and redissolve continuously. After the critical size is reached, the clusters achieve thermodynamic stability and become nuclei. This nucleation model explains only homogeneous nucleation theory where the formation of nuclei is based on supersaturation alone.

![Figure 2-5 The overall change in energy during nucleation.](image)

Based on the classic homogeneous nucleation theory, induction time \( t_{\text{ind}} \) can be correlated with the supersaturation ratio \( S \) according to the following Equation:

\[
\log(t_{\text{ind}}) = A + \frac{B}{T^3 (\log S)^2}
\]  

(2-3)
where $A$ is an empirical constant (dimensionless) and $B$ depends on a number of variables which are given below[40]. Plotting log of induction time versus $1/(\log \text{supersaturation})^2$ gives a straight line with slope equal to $B/T^3$, where $T$ is the absolute temperature in Kelvin and

$$B = \frac{\beta \gamma V_m^2 N_A f(\theta)}{(2.3R)^3} \quad (2-4)$$

Here $\beta$ is a geometric (shape) factor, $16\pi/3$ for a spherical nucleus; $f(\theta)$ is a correction factor, which is equal to 1 for homogeneous nucleation and equal to 0.01 for heterogeneous nucleation; $V_m$ is the molecular volume, 66.418 cm$^3$/mol for COM (74.6 cm$^3$/mol For COD); $T$ is the absolute temperature in Kelvin; $R$ is the gas constant in J/mol K; $\gamma$ is surface energy in J/m$^2$; and $N_A$ is Avogadro’s number in mol$^{-1}$.

The nucleation rate ($J_s$), free energy barrier ($\Delta G_{cr}$), and the radius of nucleus ($r$) can also be calculated using the following Equations:

$$J_s = F \exp \left[ -\frac{\beta \gamma V_m^2 N_A f(\theta)}{(RT)^3 (\ln S)^2} \right] \quad (2-5)$$

$$J_s = F \exp \left[ -\frac{\Delta G_{cr}}{KT} \right] \quad (2-6)$$

$$\Delta G_{cr} = \frac{4}{3} \pi r^2 \gamma \quad (2-7)$$

where $F$ is a frequency constant known as a pre-exponent factor, which has an empirical value of $10^{30}$ nuclei/cm$^3$sec, and $K$ is the Boltzman constant.

Induction time has been used to measure and compare the crystallization of calcium oxalate crystals and the effect of other urinary species on nucleation. El-Shall and his group used the relationship of induction time and supersaturation ratio discussed above to identify the effect of citrate on COM nucleation[38]. They found that citrate does not significantly change the surface energy of the COM crystal nucleate but does retard the induction time by lowering the
supersaturation through citrate complexes formed with the free calcium ions in the solution. The calculation of surface energy, nucleation rate, free energy barrier and radius of nucleus has also been widely used in other systems such as calcium sulfate[42].

Hess also used induction time to explain the inhibitory effect of citrate and the Tamm-Horsfall protein (THP) on COM crystallization by measuring the change of optical density over time[30]. Typical data can be seen in Figure 2-6. The inhibitory effect of citrate and THP was assessed by calculating the maximum slope of increase of optical density with time as the maximum rate of nucleation, $S_N$. The maximum rate of aggregation, $S_A$, was identified by the highest negative slope of optical density.

Figure 2-6 Time-course measurements of OD$_{620}$ (Optical Density at 620 nm) in a control experiment at standard conditions (calcium 5.0 mM, oxalate 0.5 mM). $S_N$, is the maximum slope of the increase of OD$_{620}$ with time, i.e. maximum rate of crystal nucleation and $S_A$ is the maximum slope of the decrease of OD$_{620}$ with time, i.e. maximum rate of crystal aggregation [30].
However, homogeneous nucleation rarely occurs in real biological systems like the urinary environment, and therefore nucleation is usually heterogeneous. The homogeneous nucleation theory discussed previously can be used to provide adequate information about the surface energy of the primary crystal nucleation in the system. Heterogeneous nucleation occurs on a foreign site present in the system, which allows the nucleation to occur at lower supersaturation levels. The foreign site in the urinary system can be either a pre-existing crystal, macromolecules such as urinary proteins, or cellular debris.

**Crystal Growth**

Every person will always nucleate calcium oxalate crystals inside kidneys due to high supersaturation of calcium and oxalate in urine. For non-stone patients, the calcium oxalate crystals are small enough (less than ~ 2 µm) to excrete out off nephrons without causing damage to the epithelial cells. Nucleation is necessary for stone formation, as it is the initiative step, but it is not the most important mechanism leading to kidney stone problems. In order for kidney stones to cause problems, crystal mass has to become large enough to be retained inside the nephrons. The growth of crystals involves a mechanism of particle coarsening called Oswald ripening. This happens when there are a variety of particle sizes in the mixer and the larger particles grow at the expense of the smaller particles in order to obtain a lower total energy of the system. Purely crystal growth is not believed to contribute much to the stone formation since the kidney filters urine at the rate of approximately around 180 L/day. The calcium oxalate crystals need to grow to the size bigger than 2 µm in less than 10 minutes to avoid getting excreted out of the body. This time interval is too short for normal crystal growth, so we can conclude that growth is not the main mechanism in the stone formation. Thus most research has been focused on the mechanism of calcium oxalate crystals aggregation and how they are retained inside the kidney.
Crystal Aggregation and Dispersion

It has been stated that stone formation is initiated by a crystal that is retained inside the kidney and grows to a stone too large to pass through the nephrons. Crystal aggregation is suspected to be a primary cause for kidney stone formation. Christmas[37] concluded that of all urinary species, citrate concentration, oxalate concentration, and proteins were the most significant variables on crystal aggregation. Fasano and Khan[15] also stated that the presence of membrane vesicles promotes crystal growth and aggregation which leads to crystal retention within the kidney. The formation of a kidney stone also involves the dispersion of the calcium oxalate crystals. Urinary proteins such as THP, bikunin, and osteopontin were among the macromolecules that have been shown to inhibit stone formation by acting as a dispersants [43]. Also, several studies showed herbal kidney stone remedies involve in increasing crystal dispersion. *Ammi visnaga* extract is also suspected to effect the dispersion of calcium oxalate crystals in the urinary system. To understand how the *Ammi visnaga* extract effects crystal aggregation and dispersion behavior, the fundamentals of crystal-crystal interaction and crystal-cell interaction should be discussed.

Crystal-crystal interaction

The mechanism of crystals aggregation and dispersion can be explained using the particle-particle interaction theory. Particle-particle interactions influence the dispersive or aggregated state of a system. If the attractive forces between particles or crystals dominate the overall forces in the system, aggregation occurs. On the other hand, dispersion or stabilization of the system occurs when the repulsive forces dominate. Several forces are involved in the particle-particle interaction and include electrostatic force, steric force, and van der Waals force[37, 43].

Electrostatic forces are developed when a solid particle is immersed in a solution resulting in the formation of surface charges. Surface charges are formed due to surface ion absorption,
surface ion imbalance, defects, or ionic exchange and dissociation of surface acids. Gouy-Chapman proposed a model of excess ionic charges on the surface of a particle and surrounding ionic species in solution, called the electric double layer. The double layer consists of the inner layer of charged ions opposite to the surface charge called Stern layer, and the outer layer called diffuse layer (Figure 2-7).

The dispersion of particles in solution depends on the separation distance from the Stern layer due to the electrostatic repulsion of the same charged layers. When particles are in a high ionic strength solution such as in urine solution, the Stern layer is compressed. The particles can then come close enough so that van der Waals force which is a short range attractive force overcomes the electrostatic repulsion force causing the particles to coagulate (Figure 2-8).

Figure 2-7 Electrical double layer.
Figure 2-8 The effect of ionic strength on the double layer. a) At low ionic strength, the double layer separation is large. b) At high ionic strength, the double layer is compressed.

The steric force is also considered to be an important factor in the kidney stone aggregation mechanism. Steric force is the physical interaction force of each molecule in the atomic scale. Urine contains some macromolecules such as the urinary proteins and lipids of which some, as the Tamm-Horsfall protein (THP), are considered to have a dispersive effect on the calcium oxalate crystals while others, such as albumin, are considered to form polymer bridging leads to crystal aggregation. Steric interaction is depended on the surface coverage of the adsorb polymers or macromolecules. The possible conformations of macromolecules that adsorb on the particle surface can be categorized to three stages depending on surface coverage (Figure 2-9).

When the surface coverage is low, polymers tend to adsorb on surface with more than one segment causing the mushroom shaped conformation where the macromolecules cluster on the adsorbed surface with no overlapping to the other particle surface. When the surface coverage is high, more polymers concentrate on the surface forcing the polymers’ chains to be close together and to extent the chain away from the surface thus causing the brush border conformations. These two conformations provide steric stabilization to the system since the adsorbed macromolecules create longer range of surface separation that can overcome the van der Waals force and add electrostatic repulsion if the macromolecules are of the same charge. However, if the surface coverage is not too low and too high, then polymer bridging between two surfaces
can occur causing particle flocculation by attaching the other end of a polymer chain to the unoccupied surface site of the other particle[43].

![Figure 2-9](image.png)

Figure 2-9 Three possible conformations of adsorbed polymer on surfaces. a) Mushroom shaped conformation at low surface coverage with no overlap with another surface. b) Bush border conformation at high surface coverage. c) Bridging between two surfaces when the coverage is not low or high.[44]

The inorganic and organic species in *Ammi visnaga* extract could impact the crystal-crystal interaction mechanisms either by changing ionic strength of the solution or changing the physical adsorbed surface. These species might complex with free ionic species in urine and increase the Stern layer separation distance. Or they could adsorb on the calcium oxalate crystal surfaces and enhance the steric stabilization of calcium oxalate crystals.

**Crystal-cell interaction**

There is some suggestion that injured renal tubular epithelia cells in kidneys are involved in the development of calcium oxalate crystal aggregates due to the increased retention force between crystal and injured cell. Epithelia cells can be injured by exposure to high oxalate concentrations or by direct scraping from sharp calcium oxalate monohydrate (COM) crystals. When the cell is injured, it releases substances like renal prothrombin fragment-1 (RPTF-1) or other anionic proteins/substances that induce the agglomeration of COM crystals leading to the formation of a stone[17]. Also, the injured cells tend to reveal the inverted side of membrane,
which is anionic, to the urinary environment. The anionic inverted membrane can act as the site for a COM crystal to adhere and grow[20, 31, 45, 46].

Verkoelen et al used the confocal microscope to study the COM crystal attachment to Mardin-Darby Canine Kidney (MDCK) cell cultures[46]. The results show COM crystals binding during the development of a confluent monolayer of the MDCK cells. Also of interest, it was seen that when injuring the cell culture, the COM crystals specifically bind to the area of injured cells during the wound healing process and none of the COM crystals bind to uninjured cells. These results confirm the hypothesis that the injured epithelia cells cause the retention of COM crystals inside the kidney and lead to the formation of kidney stones.

Figure 2-10 Calcium oxalate monohydrate crystal structure model showing calcium-rich structure on (1 0 -1) face [31].
Figure 2-11 Changes in cell membrane phospholipids and nucleation of calcium oxalate. A-a: Normal membrane with only neutral phospholipids (yellow circles) on the outer surface. B-b: Movement of acidic phospholipids (red circles) from inside to the outside. C-c: Lateral movement of acidic phospholipids into specific domains. D-d: Concentration of calcium ions and calcium oxalate nucleation on the acidic phospholipid domains [31].

Khan et al investigated the roles of lipids on the formation of kidney stones[31]. In the study, they found that the crystallization of calcium oxalate monohydrate on a Langmuir monolayer depends on the influence of lipid headgroup charges. Langmuir monolayer is a one molecule thick layer of an insoluble organic material spread onto an aqueous subphase. Most COM crystals were observed at the interfaces with anionic headgroup (glycerol > serine ≥
choline). The binding mechanism of COM crystal on the negatively charged interface can be explained by the attraction of the anionic regions to the free calcium ions in the solution by electrostatic force which resulted in regions of highly concentrated calcium. This concentrated calcium region induced the nucleation of COM crystal on the anionic lipid site and led to a crystal orientation with a calcium rich face on the lipid surface. The COM crystal structure and nucleation mechanism on Langmuir monolayer is shown below.

Figure 2-11 shows the schematic result when the cells are injured and the involvement of calcium oxalate nucleation to the change in surface phospholipids asymmetry. When the cell is injured, the apical (outer) surface looses its asymmetry, which leads to the exposure of anionic (or acidic) phospholipids from the basolateral (inner) membrane to the apical membrane. These acidic phospholipids then move to form a domain which attracts the free calcium ion to concentrate above. The high calcium concentration due to supersaturation leads to nucleation of calcium oxalate crystals on the surface. Also, the anionic phospholipid domain leads to a crystal orientation with a calcium-rich face along the phospholipids surface.

Rabinovich et al used atomic force microscopy (AFM) to measure the interaction force between silicon nitride tips-cells and COM crystal-cells[18, 19]. Two renal epithelial cell lines were used, the Madin-Darby Canine kidney epithelial cell (MDCK) and proximal tubular epithelial cells derived from pig kidneys (LLC-PK1). MDCK cells were used to represent the collecting duct kidney epithelial cell and LLC-PK1 cells were used to represent the kidney proximal tubular epithelial cell, which are the two sections on which most calcium oxalate crystals are found. There was no attractive force between the AFM tip and cells. However, when attaching a COM crystal to the AFM tip and measuring the interaction force between COM crystal and cells, they found an increased attractive force between the COM and MDCK cell
while no sign of adhesion force between COM crystal and LLC-PK1 cells. Also when investigating the interaction between COM crystal and the basal membrane (BM) of both cell lines, similar results were seen. Only the BM of MDCK cells show adhesion forces with COM crystals. When studying the effect of oxalate treated cells on the COM crystal interactions, only MDCK cells showed an increase of adhesion force with COM while the LLC-PK1 again showed no significant adhesion force. These studies provide a possible explanation on the preferential deposition of COM crystal in the collecting ducts segment (represented by MDCK cells) and lack of COM crystal appearance in the proximal tubules parts in the animal models.

Treatment of Kidney Stone

Kidney stone removal surgery is currently considered a safe and effective procedure for treating kidney stone patients. However, more than 50% of kidney stone patients face the problem of kidney stone recurrence and have to repeat the surgery over and over again. Going through several surgical treatments to remove kidney stones could be painful, frustrating, and costly. Several methods are developed in order to avoid kidney stone surgery for the patient. Extracorporeal shockwave lithotripsy, oral supplements and herbal remedy are effective alternatives.

Extracorporeal Shockwave Lithotripsy

Extracorporeal shockwave lithotripsy (ESWL) is a method using sound waves to break the kidney stone to small pieces. When the stone breaks into small pieces, it can be excreted out of kidney safely without surgery. However, ESWL does not prevent the recurrence of kidney stones; therefore, patients who reform kidney stones have to go through ESWL repeatedly. The method of ESWL also involves a huge water-sonicated pool which is less convenient than taking oral supplements. Long term treatment of ESWL may cause hypertension and could lead to chronic kidney failure. The fragments of stone that remain in the kidney could be sites for
heterogeneous nucleation of new stones. Also, repeated treatments of ESWL can be costly and time consuming.

**Oral Supplements**

Several oral supplements have been given to the kidney stone patients as an aid for preventing the recurrence of kidney stones. The well-known supplements for kidney stone treatment mostly contain citric acid and magnesium salt.

Citrate is known to be one of the inhibitors of kidney stone formation. Citrate in human plasma exists as an alkaline citrate ($C_6H_5O_7^{3-}$) in a range from 0.05-0.3 mmoles/liter\[47\]. The abnormal level of citrate in human urine, known as hypocitraturia, is one of major contributors to kidney stone disease besides hypercalciuria and hyperoxaluria. Hypocitraturia is a symptom that urinary citrate is decreased from the normal level by enhanced reabsorbance by renal tubules and impaired uptake\[48\]. Due to the anionic charge of alkaline citrate, it competitively binds free calcium ions in urine so that there are fewer available free calcium ions to interact with oxalate ion and form calcium oxalate crystals. The reactions below show such mechanism.

$$Ca^{2+}(aq) + C_2O_4^{2-}(aq) + n H_2O \leftrightarrow CaC_2O_4.nH_2O(aq)$$

$$Ca^{2+}(aq) + C_6H_5O_7^{3-} \leftrightarrow Ca_3(C_6H_5O_7)_{2}(aq)$$

$$CaC_2O_4.nH_2O(aq) \leftrightarrow CaC_2O_4.nH_2O(s)$$

The solubility of calcium citrate is higher than the solubility of calcium oxalate in any hydration state. Therefore, the calcium citrate complex stays in aqueous solution while the calcium oxalate aqueous complex precipitates to solid crystals; thus citrate inhibits nucleation. Pak et al. showed that the treatment of hypocitraturia slowed down or eliminated kidney stone formation [7, 49]. In vitro studies have shown citrate to inhibit precipitation of calcium oxalate crystal [30, 38]. Also, citrate has been shown to prevent aggregation and growth of calcium oxalate [9].
Potassium citrate has been given to kidney stone patients as an oral supplement to treat hypocitraturia by physicians because of the inhibitory effect of citrate stated above[50]. Sodium citrate is also used, however not as frequently since high sodium intake may increase urinary calcium excretion.

Magnesium salt is another main oral supplement used for kidney stone disease. Magnesium ion has the same positive charges as calcium ion. Therefore, magnesium ions competitively bind free oxalate ions the same way as citrate does to calcium ions. Several studies have proved the effectiveness of magnesium in preventing or reducing the recurrence of kidney stones in both rat and human subjects. Hammarsten et al. studied the effect of MgCl₂ on the solubility of calcium oxalate in water. They found out that addition of MgCl₂ in the solution increases the solubility of calcium oxalate[51]. Gershoff et al. showed that when adding magnesium in a kidney stone-induced rat subject, the deposition of calcium oxalate crystal to the kidney was reduced[52]. Also they tested with 30 human stone former subjects and found out that MgO oral supplement helped reducing or eliminating the recurrence of kidney stones. Leiske et al. studied the effect of magnesium on the interaction between calcium oxalate monohydrate crystals and kidney epithelial cells. They measured the number of ¹⁴C COM crystals that stuck to cells after being treated with Mg²⁺ and Ca²⁺ ions and rinsed with buffer solution. They found that there were maximal adhesions of COM crystals to the MCDK cells at the critical concentration of Ca²⁺ and Mg²⁺, above the normal physiological concentration in urine[53].

Significance of Herbal Medicinal Treatment of Kidney Stone

Herbs Related to Kidney Stone Treatment

Herbal remedies of kidney stone have been known for many centuries and used all over the world. Also, herbal remedies or “folk medicine” are still widely practiced in the Mediterranean, Middle East, and Asia. Several plant extracts have been identified as a diuretic and remedy for
kidney stones. Atmani et al. investigated *Herniaria hirsuta* on calcium oxalate crystallization *in vitro* and *in vivo* [28, 54]. *H. hirsuta* has been widely used to treat kidney stone symptoms in the Mediterranean area. Also in Japan, *Alismatis rhizome*, or Takusha, is used in traditional Japanese herbal therapeutics for the treatment of stone disease [55]. *Bergenia ligulata* is used in India and *Phyllanthus niruri* in Brazil [56, 57]. Several investigators have studied the effect of the plants mentioned above on stone formation. Similar results have been seen in these studies. The extract induces the nucleation of the calcium oxalate crystals; however, the crystals morphology is most often present in the dihydrate form instead of monohydrate form. Also the size of nucleated crystals becomes smaller as the plant extract concentration increases. Moreover, some plants extracts show dispersive activity on the calcium oxalate crystal leading to less aggregation. All studies show high potential of the herbal extracts for the reduction of the stone reoccurrence, even though the plant extracts may lead to the nucleation of more crystals. The generation of smaller, less stable calcium oxalate dihydrate crystals by the plant extract, leads to nephrons easily passing the crystals out the urinary tract.

**Ammi visnaga (Khella) Extract**

In addition to all of the plant extracts mentioned above, *Ammi visnaga* (Khella) is reported as an effective traditional remedy for kidney stone in Egypt as mentioned back in the Ebers papyrus (c 1500 BC) [10]. *Ammi visnaga* is a tall plant with thick stem and small leaves grown in the Middle East around the Mediterranean and in South Africa [58]. The flower is white with seed inside. The extract is normally prepared by the infusion method or the decoction method to make herbal tea from dried seed. The herbal tea is given to the patients orally for a diuretic of kidney stone and to relieve pain related to the renal stone disease. The key constituents of *Ammi visnaga* are khellin, visnagin, khellol glycoside, volatile oil, flavonoids, and sterols [10, 11]. *Ammi visnaga* extracts are also used as antispasmodic, antiasthmatic and muscle relaxant agents.
The two key constituents of *Ammi visnaga*, khellin and visnagin, were believed to be the active constituents of the plant [11]. Both khellin and visnagin are the derivative form of coumarins, which has benzopyrone as a core skeletal structure. The structures of khellin and visnagin are shown in Figure 2-12.

![Structure of khellin and visnagin](image)

**Figure 2-12 Structure of khellin and visnagin**

Khan Z.A. et al. concluded that a possible explanation for the diuretic activity of *Ammi visnaga* extract was because it prevents precipitation of the calcium oxalate crystal, even though the concentration of oxalate is sufficient. In the study, they also found that *Ammi visnaga* treated rats had less calculi deposition in their kidney compared to the glycolic acid control group [59]. Rauwald et al. suggested that the active compounds in *Ammi visnaga* fruits, khellin, visnagin, and visnadin could be involved with the Ca$^{2+}$ channel blocking [60]. *Ammi visnaga* seeds also have an antispasmodic effect on the muscles which reduces the pain from the trapped kidney stone and helps ease the stone down the bladder. From these studies of crystal-cell interaction, it can be seen that crystal retention inside the kidney is connected with the cell injury mechanism. *Ammi visnaga* extract has been reported to ease the stone down to the bladder which means that the active constituents in the extract could also reduce the crystal-cell adhesion force or impact the releasing of substances during the cell injury itself.
Characterization Techniques

Characterization techniques used in this research include high performance liquid chromatography (HPLC), inductively coupled plasma (ICP), light absorbance measurement by UV-Vis spectrometer, and atomic force microscopy (AFM) and these are discussed below.

High Performance Liquid Chromatography (HPLC)

High performance liquid chromatography is used to identify the presence of organic components such as khellin and visnagin in Khella extract. HPLC is developed from column chromatography, where high pressure is used to force liquid solution (mobile phase) through a column packed with stationary phase. Small volume of the sample is injected through the column with a stream of mobile phase. The separation of components in the sample depends on physical and chemical interaction between the component, mobile phase, and the stationary phase in the column. For each component, the time that it comes out at the end of the column is called retention time. Retention time is a unique characteristic for each specific component so it is used for identification methods. High pressure that is used in HPLC increases the velocity of liquid flow through the column; therefore, it reduces retention time and improves the resolution when compared with regular column chromatography and thin layer chromatography. The mobile phase composition can be varied during an analysis for higher resolution using a method called gradient elution. The gradient elution program controls the amount of each chemical in the mobile phase as a function of time. This allows the HPLC to separate components as a function of mobile phase composition which helps refine the resolution. The mobile phase composition depends on the nature, physical and chemical interaction to stationary phase, of each component that needs to be separated.

Mobile phases used for determination of khellin and visnagin in Khella extract by several research groups were mixture of water, acetonitrile, tetrahydrofuran (THF), ethanol, and
methanol in different ranges of concentration depending on the type of stationary phase of column. The composition of the mobile phase in this study is developed based on what has been done previously and the composition that maximized the retention time peak resolution is selected as described later in the material and methods section.

**Inductively Coupled Plasma (ICP)**

Inductively coupled plasma is used to determine free ions of single elements such as calcium, potassium, and magnesium in Khella extract. The ICP spectrometer uses of an argon plasma torch at high temperature in the order of 10,000 K. When the droplet of sample passes the plasma torch, the plasma evaporates any solid components that dissolved in the solution and breaks them down to atoms. The charge from the atoms from the sample is measured. The concentration of each single element ion in the sample is calibrated with the element standard solutions.

**UV-Vis Spectrometer**

UV-Vis spectrometer is equipment used to measure the absorption of ultraviolet and visible light after it passes through a sample or after it reflects on a sample surface. To measure the nucleation, absorption of selected a single wavelength is used and is measured as a function of time. The Beer-Lambert law provides the relationship between analyte concentration and absorbance:

\[ A = \varepsilon bc \]  \hspace{1cm} (2-8)

where \( A \) is an absorbance, \( \varepsilon \) is the molar absorptivity, \( b \) is the path length of the sample, and \( c \) is the concentration of compound in solution.

Therefore, the absorbance increases as a mixture nucleates crystals which reflect or absorb light. The absorbance is measured relative to a control solution in a cuvette. The solution with particles will have less light transmittance or higher light absorbance.
Atomic Force Microscopy (AFM)

Atomic force microscopy (AFM) is used to measure the interaction force between a sample and an AFM tip on an atomic level. An AFM tip, the sharp end of a cantilever, is normally made of silicon or silicon nitride. However, sometime the tip can also be modified by attaching a particle or polymer in order to measure the interaction force between a particle and a sample. The interaction force is measured by the movement of the cantilever that is caused by repulsive or attractive force between the tip and the sample. The sample is placed on a piezoelectric scanner and then slowly rises to the AFM tip. The up or down movement of the cantilever due to repulsive or attractive force is detected by the movement of a laser path which points directly to the AFM tip. When the tip reflexes, it causes a laser beam to move and this movement is detected by a photo-detector. The schematic of AFM is shown in Figure 2-13.

Figure 2-13 Atomic force microscopy
In this study, AFM is used to measure the interaction between a single COM crystal and kidney epithelial cell surface. A single COM crystal is attached to an AFM tip and the measurement is done in a liquid chamber. The urinary species are added to the buffer solution to study the effect of those species on crystal-cell interaction.
CHAPTER 3
MATERIALS AND METHODS

Extract Preparation

Seeds were obtained from two sources, one in Egypt and one in Turkey. The Egyptian Khella seeds were obtained from herbalist in Egypt. Turkish seeds were obtained from Caelo (Caesar & Lorentz GmbH, Hilden, Germany) and certified to be *Ammi visnaga*.

Extracts were prepared from seeds of Khella by hot water infusion. 20 g of dried ground seeds were added to 200 ml of 100 ºC nanopure deionized water. The mixtures were left at 100 ºC for 5 minutes. The decoctions were left to cool at room temperature. Following cooling, the mixtures were filtered through a 0.45 µm membrane (Fisherbrand, Pittsburgh, PA) and the supernatants were kept. Both supernatants were freeze-dried overnight. The dried flakes were collected and kept at – 20 ºC for further use.

The freeze-dried flakes of extract were collected and weighted to be approximately 3.5 g per 200 ml of extract decoction prepared. Then 0.18 mg of freeze dried flakes of Khella extract were dissolved in 1 ml of deionized nanopure water so that the final concentration for each experiment was equivalent to 1 to 100 dilution of the original extract decoction (1 mg of dried seed per 1 ml of deionized water).

Extract Characterization

Thin Liquid Chromatography (TLC)

Organic components of the extracts were identified using thin layer chromatography (TLC) and high performance liquid chromatography (HPLC). TLC was used as a quick screening on whether the extracts contained khellin or visnagin or not. 0.5 mg of dried flakes were added to 5 ml of 60% ethanol. 5 mg of standard khellin and visnagin were added to 1 ml of 60% ethanol. All solutions were filtered through 0.2 µm filter paper (Fisherbrand, Pittsburgh, PA). The
solutions were then transferred to TLC paper using capillary tubes. The TLC paper was marked at 1.5 cm line, so that the travel distance for the liquid chromatogram was 10 cm (Figure 3-1).

After transferring all the solution at the 1.5 cm line using capillary tubes, the TLC paper was merged into ethyl acetate solution that had not reached the 1.5 cm drop marked. Then the TLC paper was left until the ethyl acetate mobile phase reached the 10 cm line marked. To identify khellin and visnagin in the extracts, the TLC paper was exposed to UV- light. Spots of khellin and visnagin in the extract should match the standard khellin and visnagin.

Figure 3-1 The TLC paper preparation.

**High Performance Liquid Chromatography (HPLC)**

HPLC was done to confirm the TLC results and quantify the amount of khellin and visnagin in the extract. The analysis was performed using a Shimadzu liquid chromatograph (LC-10 AT) with a ternary solvent delivery system, combined with a Diode Array detector (SPD-M 10 A), and a Rheodyne loading valve fitted with a 100 µl sample loop. The HPLC
column used for khellin and visnagin separation in *Ammi visnaga* extract was a LiChroCart RP-select (250 mm x 4 mm i.d., 5 µm particle diameter) reversed-phase column, with a pre column (4.5 mm i.d. x 2.5 cm) containing the same packing. Separation of khellin and visnagin was done by using the gradient elution program and used the following solvents for the mobile phase: A was deionized water, B was Tetrahydrofuran, THF, (Fisher Science, HPLC grade), and C was methanol (Fisher Science, HPLC grade). All solutions used for the mobile phase were filtered through a 0.45 µm filter (Fisherbrand, Pittsburgh, PA) and then degassed using an ultrasonicator. The gradient applied was as follows: 5 to 13% B from 0-20 minutes with constant 5% C and balanced with A, 13 to 22% B and 5 to 7% C from 20-52 minutes balanced with A, and then 22-5% B and 7-5% C from 52-60 minutes balanced with A. 10 µl of each sample, extract solutions, khellin standard and visnagin standard, were injected and the detection of peaks was recorded at 330 nm (optimized wavelength). HPLC analyses were carried out at a constant temperature of 30 ºC. The mobile phase flow rate was 1 ml/min. To quantify the amount of khellin and visnagin in the extract solution, several standardize mixtures of pure khellin and visnagin (Sigma Aldridge) in the range of 0.05-1 mg/ml were used in the HPLC experiments to obtain a calibration curve. The amount of khellin and visnagin in the seed extracts was quantified using the areas of the peaks.

**Inductively Coupled Plasma (ICP)**

Calcium and magnesium ions in the extracts were identified using inductively coupled plasma (ICP, Perkin Elmer Optima 3200RL Optical Emission Spectroscopy, Norwalk, CT). The instrument was calibrated using 0 ppm (nanopure water), 1 ppm, 10 ppm, and 100 ppm calcium (calcium reference solution certified 1000 ppm ± 1%, Fisher Scientific, Fair Lawn, NJ) and magnesium (magnesium reference solution certified 1000 ppm ± 1%, Fisher Scientific, Fair Lawn, NJ) solution and evaluated using non-linear regression. For each sample, 5 readings were
taken at all wavelengths for calcium and magnesium defined in the program. The pump flow rate was 1 ml/min and delay time was 60 seconds.

**Oxalate identification**

Since oxalate is not single element components, ICP could not be used to analyze both components.

To determine oxalate concentration of extracts, a modified microassay from an oxalate kit was used (591-D; Trinity Biotechnology, St. Louis, MO). Briefly, 100 µl of extract was mixed with 100 µl of sample buffer (supplied with kit). The 1:1 mix was then added to activated carbon in a 1.5 ml tube (supplied with kit). The tube was then mixed on a rotator for 5 minutes. The tubes were then centrifuged at 13000 rpm in a microcentrifuge. 10 µl of clear supernatant was placed into a designated well of a 96 well plate. 10 µl of 0.25 µM, 0.50 µM, and 1 mM oxalate standards (supplied with kit) were place in designated wells. Reagent A was then added and then Reagent B (both supplied with kit) to all wells. The plate was gently shaken for 10 minutes at room temperature. The plate was read using a Bio-Rad Microplate reader at 595 nm.

**Nucleation Study**

**Induction Time Measurement**

The nucleation mechanism of calcium oxalate crystals can be determined by using optical density measurements. A UV-Vis spectrometer (Perkin-Elmer, Lamda 800) was used to measure the light absorbance as a function of time. The induction time, time that detectable crystal can be identified, was estimated from the point of maximum slope of light absorbance (Figure 3-2).

A wavelength of 560 nm (optimized wavelength) was used to measure the change in light absorbance of each mixture in 4ml cuvettes. The light absorbance was measured every 5 seconds for 30 minutes. The UV-Vis spectrometer sample cuvette holder was attached to a heated water pump via rubber tubing to maintain the sample temperature at 37º C.
Figure 3-2 Induction time approximation from UV-Vis light absorbance graph.

The experiment was designed to study the effect of some of the urinary species and of Khella extract and its components on the induction time at different supersaturation ratios of calcium and oxalate solutions. The supersaturation ratio was calculated based on Equation 2-1 which was given in Chapter 2. The range of supersaturation ratios was selected to be 2.0 to 2.8. The induction time of other supersaturation ratios that were out of this range were obtained by extrapolating the data using linear relationship between natural log of induction time and natural log of supersaturation ratio.

**Membrane Isolation**

The LLC-PK1 and MDCK cell membrane debris were prepared and isolated with the help of Karen Byers from Dr. Khan’s Pathology lab. The membrane isolation method used was based on the rate zonal sucrose gradients according to a standard published protocol [61, 62]. The cells were homogenized with isolation buffer (300 mM D-Mannitol, 5 mM EGTA, 12 mM Tris HCL, pH 7.4) using a handheld motorized homogenizer (Fisher brand tissue tearor) for 10-20 seconds. 1.5 mL of 120 mM MgCl was added to the homogenized tissue mixture and shaken for 20 minutes on ice.
Then the mixture was centrifuged at 4500 rpm for 15 minutes. The supernatant and pallet were separated and saved. The pallet was homogenized and centrifuged again with the same method stated above. Then the supernatant was collected and combined with the supernatant from the previous spin. The supernatant was centrifuged again at 16000 rpm for 30 minutes. The pallet was collected after this spin. Then the pallet was homogenized again with 30 ml of ½ strength isolation buffer. Then 3 mL of 120 mM MgCl was added to the mixture. The mixture was shaken for 20 minutes on ice then centrifuged at 4500 rpm for 15 minutes. The supernatant was collected after this spin. Then supernatant was centrifuged at 90000 rpm for 60 minutes at 4 °C. After centrifugation, the supernatant was separated into layers containing different parts of cells (Figure 3-3). The layer of brush border membrane was gently pulled out using a micropipette. The content of membrane in the solution was determined using a Lowry protein essay.
Solution Preparation

Solutions of calcium chloride (CaCl$_2$, A.C.S. Grade, 74%-78% assay, Fisher Scientific Inc.) and potassium oxalate (K$_2$C$_2$O$_4$, A.C.S. Grade, 100.8% assay, Fisher Scientific Inc.) were prepared at four times the concentration for each supersaturation ratio. For the control experiment 1 ml of calcium chloride, 1 ml of potassium oxalate, and 2 ml of deionized water were mixed together so the final concentration of the mixture matched the supersaturation ratio shown in Table 3-1 below.

<table>
<thead>
<tr>
<th>Supersaturation Ratio</th>
<th>[CaCl$_2$] mM</th>
<th>[K$_2$C$_2$O$_4$] mM</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.0</td>
<td>0.43</td>
<td>0.43</td>
</tr>
<tr>
<td>2.2</td>
<td>0.473</td>
<td>0.473</td>
</tr>
<tr>
<td>2.4</td>
<td>0.516</td>
<td>0.516</td>
</tr>
<tr>
<td>2.6</td>
<td>0.559</td>
<td>0.559</td>
</tr>
<tr>
<td>2.8</td>
<td>0.602</td>
<td>0.602</td>
</tr>
</tbody>
</table>

To study the effect of each urinary species, solutions of each species were prepared in deionized water at concentration of four times the desired final concentration. Each run, 1 ml of calcium chloride, potassium oxalate, deionized water, and urinary species of interest were mixed in a 4 ml cuvette and tested for the change of light absorbance. The final concentration of each species is shown in Table 3-2.

<table>
<thead>
<tr>
<th>Species</th>
<th>Concentration</th>
<th>Unit</th>
</tr>
</thead>
<tbody>
<tr>
<td>Egyptian Khella extract</td>
<td>0.1</td>
<td>mg (dry seed)/ml (water)</td>
</tr>
<tr>
<td>Turkish Khella extract</td>
<td>0.1</td>
<td>mg (dry seed)/ml (water)</td>
</tr>
<tr>
<td>Khellin</td>
<td>0.03-0.12</td>
<td>mg/ml</td>
</tr>
<tr>
<td>Visnagin</td>
<td>0.005-0.03</td>
<td>mg/ml</td>
</tr>
<tr>
<td>Citrate/ [Mg$^{2+}$]</td>
<td>0.1</td>
<td>mM</td>
</tr>
<tr>
<td>MDCK cell debris</td>
<td>10</td>
<td>µg/ml</td>
</tr>
<tr>
<td>LLCPK cell debris</td>
<td>10</td>
<td>µg/ml</td>
</tr>
<tr>
<td>Bovine Serum Albumin</td>
<td>15</td>
<td>ppm</td>
</tr>
</tbody>
</table>

Table 3-2 Final concentration of each species tested for light absorbance measurement.

To study the effect of each urinary species, solutions of each species were prepared in deionized water at concentration of four times the desired final concentration. Each run, 1 ml of calcium chloride, potassium oxalate, deionized water, and urinary species of interest were mixed in a 4 ml cuvette and tested for the change of light absorbance. The final concentration of each species is shown in Table 3-2.
The relationship between induction time and supersaturation ratios was used to calculate surface energy of nuclei and the free energy barrier based on the classical homogeneous nucleation theory mentioned in chapter 2.

**Calcium Titration Measurement**

To study the interaction of calcium and oxalate in solution, the amount of free calcium ions in solution was checked using the calcium electrode attached to a TIM 856 Titration Manager Radiometer. The amount of calcium ion in solution was measured as the potential reading in voltage which was converted to concentration using a calibration curve. Higher voltage means there is higher amount of calcium in solution. The titration measurement was recorded as a function of time. The depletion of calcium in solution means that there is less free calcium available in solution due to precipitation or calcium binding of the component of interest.

**Crystal Morphology and Crystallinity**

To check the effect of Khella extract on calcium oxalate morphology, the crystals formed after 30 minutes of light absorbance measurement were collected by filtering the mixture through 0.2 µm filter paper (Fisherbrand, Pittsburgh, PA). The crystals were transferred to carbon tape and attached to ½-inch scanning electron microscope aluminum stages. The samples were coated with gold using a plasma sputtering system (IB2 Ion Coater, Eiko Engineering) for 1 minute and examined with a field emission scanning electron microscope (JEOL 6330F). The presence of crystal was verified and crystals were identified morphologically. The compositions of crystals were examined using EDS. Also SEM pictures provided image analysis of the average size distribution of calcium oxalate crystals formed from each experiment.

The morphology of COM crystals, used for AFM studies, was also verified using the SEM method described above. Also, to confirm the hydration state of calcium oxalate crystals formed, energy dispersive x-ray was used.
Crystal-Cell Interaction Study

Preparation of COM Crystals for AFM Study

COM particles used in the AFM study were grown by homogeneous nucleation in order to obtain a single COM particle with no twinning and larger than 10 µm in diameter. All glassware was acid washed. Water used in this experiment was nanopure deionized water. One liter of two solutions, $10^{-3}$ M CaCl$_2$ (CaCl$_2$, A.C.S. Grade, 74%-78% assay, Fisher Scientific Inc.) and $10^{-3}$ K$_2$C$_2$O$_4$ (K$_2$C$_2$O$_4$, A.C.S. Grade, 100.8% assay, Fisher Scientific Inc.) were made in a buffer of 0.1M NaCl (NaCl, A.C.S. Grade, 99.0% assay, Fisher Scientific Inc.). The solutions were filtered through a 0.2 µm filter paper (Fisherbrand, Pittsburgh, PA). Then both solutions were stored in an incubator to equilibrate to physiological temperature, 37°C, for 24 hour. The two solutions were slowly mixed together in the incubator by dropping the K$_2$C$_2$O$_4$ solution into the CaCl$_2$ solution over the magnetic stirrer (Figure 3-4).

In the incubator set at 37°C, the calcium chloride solution was transferred to a 2 liter beaker and stirred in medium speed. The potassium oxalate solution was added to the calcium chloride solution using a titration pipette at the rate of approximately 1 l/hr. After adding all the potassium oxalate solution to the calcium chloride solution, the mixed solution was left unstirred in the incubator for 22 hours.

After both solutions were completely mixed, the stirrer was turned off and the mixed unstirred solution was left in the incubator at 37°C for at least 22 hours. The crystals formed after 22 hours were collected by vacuum filtration with a 10 µm filter paper (Fisher NYL Membrane 10 µm CAT# R99SP04700). The filter residues were washed thoroughly with COM saturated solution to extract the smaller size crystals. The filtered COM particles were washed
with saturated COM solution to reduce dissolution of particle and then freeze-dried. The dried particles were kept at -20°C for further use.

Figure 3-4 Instrument setup for mixing solution in the incubator to prepare COM crystals at 37 °C.

Artificial Urine Solution Preparation

Artificial urine solution was prepared according to compositions shown in Table 3-3[36]. However, an artificial urine solution used in all experiments in this study has been altered to leave out sodium citrate, calcium chloride, and sodium oxalate. These component concentrations were adjusted and added later to match desired concentrations for the designed experiments.

All the components were added to nanopure water in the order shown in Table 3-3. Then the solution was filtered by vacuum filtering (0.22 μm Nylon, Osmonics Inc.) and stored at 4 °C
for up to a week. Before each AFM experiment, the artificial urine solution temperature was
adjusted to 37 °C. The pH of the artificial urine solution was adjusted to 6.6 using sodium
hydroxide or hydrochloric acid.

Table 3-3  Total concentration of artificial urine displayed by the order of addition.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Solution Concentration (M)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl</td>
<td>0.10554</td>
</tr>
<tr>
<td>NaH₂PO₄•2H₂O</td>
<td>0.03654</td>
</tr>
<tr>
<td>MgSO₄</td>
<td>0.00385</td>
</tr>
<tr>
<td>Na₂SO₄</td>
<td>0.01695</td>
</tr>
<tr>
<td>KCl</td>
<td>0.06374</td>
</tr>
<tr>
<td>NH₄Cl</td>
<td>0.03632</td>
</tr>
<tr>
<td>NH₄OH</td>
<td>0.00062</td>
</tr>
</tbody>
</table>

**Cell Culture**

Two cells lines were selected to use in this study, LLC-PK1, a proximal tubular cellular
membrane derived from porcine kidney, and MDCK, a collecting duct cellular membrane
derived from canine kidneys. Both LLC-PK1 and MDCK cell lines have microvilli brush border
and form continuous monolayer in culture with uniform cell-to-cell contacts. The difference in
structure of microvilli between the two cell lines was discussed earlier in chapter 2.

Mardin-Darby Canine Kidney (MDCK) and Porcine Kidney (LLC-PK1) cells were
obtained from American Type Culture Collection (ATCC, CCL-34 and CL-101; Manasses, VA).
Cells were maintained as continuously growing monolayer in culture in growth media (1:1 ratio
Dulbecco’s modified essential medium nutrient mixture and F-12 (DMEM/F-12, Gibco BRL,
Grand Island, NY) containing 10% newborn calf serum, 15 mM HEPES, 20 mM sodium
bicarbonate, 0.5 mM sodium pyruvate, 17.5 mM glucose, streptomycin and penicillin) in 75 cm²
Falcon T-flask (12-565-52; Fisher, Norcross, GA) at 37 °C in a 5% CO₂ air atmosphere
incubator. Under these conditions, cells achieved confluence. Cells were subcultured by
disassociation with 0.05% trypsin and 0.25% ethylenediaminetetraacetic acid (EDTA) and
seeded to 12-well plates (07-200-82; Fisher, Norcross, GA) containing Thermanox slide (NC9068818; Fisher, Norcross, GA) attached to an AFM aluminum stage. After reaching confluence, growth media was removed by acclimatization media (500 ml DMEM/F-12, 10 ml of antibiotic-antimyotic solution, 5 ml of insulin/transferring/selenium mix, 1 ml of hydrocortisone, 3.4 ml of tiiodo-L-thyonine, 1 ml of prostaglandin E1) for 8 to 12 hours.

The acclimatization media were removed and replaced with artificial urine right before AFM measurements. When transferring the cells to the AFM liquid chamber, small amount of artificial urine solution remained on the top of the cells to keep the cells from drying out and dying.

**Direct Force Measurement between COM Crystal and Kidney Renal Cells Using AFM**

An atomic force microscope was used to measure the direct force interaction between COM crystal and kidney epithelial cell membrane. A single COM crystal was glued (Epon R Resin 1004 F from Shell Chemical Co) to a silicon nitride AFM tip by using a tri-axial micromanipulator unit attached to an optical microscope. The force measurements were obtained following the method by Ducker and Senden [63]. The AFM tip with COM crystal is shown below in Figure 3-5.

An atomic force microscope (AFM) with a Nanoscope III controller form Digital Instruments, CS, was used for the force measurements and operated by Dr. Yakov Rabinovich. Triangular cantilevers (NP-S type) from Veeco Instruments, CA, with a normal spring constant of 0.12 N/m were used. The single COM crystal attached to the AMF tip had average size of 14-16 µm. All the force measurements were carried out in the standard liquid chamber of the AFM. All measurements were in artificial urine solution or Tris buffer solution with different concentrations of compounds in questions.
To study the effect of Khella on the interaction between COM crystal and MDCK cells, the AFM measurement was done in artificial urine solution (AUIS) medium as a control experiment and in AUIS with 1mg/ml of Egyptian Khella extract.

Figure 3-5 An AFM tip attached with a single COM crystal used for crystal-cell force measurement.
CHAPTER 4
RESULTS AND DISCUSSION

Khella Extract Active Component Characterization

Several characterization techniques (see materials and methods) were used to identify both inorganic and organic components in the extract solution.

**Inorganic Component Identification**

An inductively coupled plasma (ICP) was used to identify single inorganic components, such as calcium, and magnesium in both Egyptian and Turkish freeze dried extract solution. The amount of each element is shown in Table 4-1.

<table>
<thead>
<tr>
<th>Type of Extract</th>
<th>Concentration (mg/L)</th>
<th>Extract Dried-flake</th>
<th>Mg$^{2+}$</th>
<th>Ca$^{2+}$</th>
<th>K$^+$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Egyptian Khella</td>
<td>180</td>
<td>1.88</td>
<td>2.9</td>
<td>13.84</td>
<td></td>
</tr>
<tr>
<td>Turkish Khella</td>
<td>180</td>
<td>1.22</td>
<td>8.34</td>
<td>11.11</td>
<td></td>
</tr>
</tbody>
</table>

The ICP results show that both extracts contained magnesium, calcium, and potassium which are reported in the literature to affect nucleation of calcium oxalate crystals. The magnesium and potassium levels in the two extracts are relatively close, while the calcium content of the Turkish Khella extract is triple that of the in Egyptian extract.

Oxalate concentration was determined using the oxalate kit from trinity biotechnology. Both extracts contain relatively insignificant amount of oxalate (approximately around 0.01mM).

**Organic Component Identification**

The organic active components in Khella extract, such as khellin and visnagin, were identified using thin layer chromatography (TLC) and high performance liquid chromatography (HPLC).
**Thin liquid chromatography (TLC)**

The TLC method was used to do a quick screening on the organic component content of each extract. TLC showed no khellin and visnagin in the Egyptian extract while there were orange/brown spots, which match the standard khellin and visnagin, shown in the Turkish extract.

**High performance liquid chromatography (HPLC)**

HPLC experiments were done to confirm the TLC results and also to quantify the concentration of each component in the extract. Using a pure standard of khellin and visnagin in HPLC, the peaks of khellin and visnagin appear at 29 minutes and 33 minutes respectively (Figure 4-1). Several concentrations of standard khellin and visnagin were used in the HPLC experiments to obtain the calibration curve that can be used to estimate the amount of khellin and visnagin in the seed extracts from area of the peak (Figure 4-2).

![HPLC graph of 0.5 mg/ml of pure standard khellin and visnagin](image)

**Figure 4-1** HPLC graph of 0.5 mg/ml of pure standard khellin and visnagin

- **Visnagin @ 33 min**
- **Khellin @ 29 min**
Figure 4-2 Calibration curve for determination khellin and visnagin concentration from HPLC peak area.

Figure 4-3 HPLC graph of Egyptian Khella seed extract.

- Small peak around 29 min and 33 min
- Checked the chromatogram
  - Not matched with pure standard
  - So the extract does not contain Khellin and Visnagin
The HPLC shows that the extract from Egyptian Khella seeds contained no khellin or visnagin while the Turkish *Ammi visnaga* certified seed extract shows peaks for khellin and visnagin. This result correlates well with the khellin and visnagin identification by TLC.

The reason that Egyptian Khella seeds extract shows no sign of khellin and visnagin might be because of improper storage at the local herbalist which leads to the degradation of khellin and visnagin compounds in the seed. It is also possible that the Khella seeds received from Egypt are not *Ammi visnaga* but a close relative species called *Ammi majus* [11].

As seen in Figure 4-3, the Egyptian Khella seed extract has small peaks at the separation time of khellin and visnagin. However, both peaks are very small. From this it can be concluded that the extract do not have any significant amount of khellin and visnagin present. The Turkish *Ammi visnaga* extract shows clear peaks of khellin and visnagin and matches the chromatogram.
of the standards. Three repetitions of the HPLC experiments were performed for the quantification of khellin and visnagin in Turkish Ammi visnaga seed extract (Figure 4-4). The amounts of khellin and visnagin estimated using the calibration curve in Turkish Ammi visnaga seed extract (1 g of dry seed/ 10 ml of water) are 0.42 mg/ml and 0.18 mg/ml, respectively.

Even though the Egyptian extract does not show the presence of khellin or visnagin which according to the literature are the active components, it is being used by herbalists for the treatment of kidney stones. Therefore, both seed extracts are tested in our studies. There might be other active components that are common in both extracts which could be active components that effect calcium oxalate crystallization.

**Effect of Khella Extract on Calcium Oxalate Crystallization**

**Effect of Khella Extract on Nucleation of Calcium Oxalate Crystals**

A nucleation study on the effect of Khella was conducted as it is the first step of crystallization. Both Khella extracts were tested and compared with pure calcium and oxalate control solutions. The responses on nucleation mechanism tested included induction time, crystal morphology, surface energy, nucleation, and aggregation rate approximation, and Gibbs free energy calculation.

**Light absorption measurement**

A UV-Vis spectrometer was used in the nucleation study to measure the induction time of crystals formation and to estimate nucleation, and aggregation rate. Induction time, nucleation rate and aggregation rate can be estimated from the plot of light absorbance as function of time (Figure 2-6 and Figure 3-2). An example of the effect of Khella extract on light absorbance measurement is shown in Figure 4-5. The induction time at each supersaturation ratio was estimated and the values are shown in Table 4-2. The standard deviations from triplicates are also shown in Table 4-2.
Figure 4-5 Effect of Khella extract on light absorbance at initial supersaturation of 2.0.

Table 4-2 The effect of Khella extract on induction time of calcium oxalate nucleation from UV-Vis light absorbance measurement at various supersaturations.

<table>
<thead>
<tr>
<th>SS</th>
<th>Control</th>
<th>Induction time (s) with Egyptian Khella</th>
<th>Induction time (s) with Turkish Khella</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.0</td>
<td>376±10</td>
<td>N/A</td>
<td>675±18</td>
</tr>
<tr>
<td>2.2</td>
<td>128±43</td>
<td>289±9</td>
<td>252±8</td>
</tr>
<tr>
<td>2.4</td>
<td>88±8</td>
<td>188±23</td>
<td>377±58</td>
</tr>
<tr>
<td>2.6</td>
<td>74±5</td>
<td>126±32</td>
<td>85±30</td>
</tr>
<tr>
<td>2.8</td>
<td>38±2</td>
<td>78±27</td>
<td>111±24</td>
</tr>
</tbody>
</table>

The results show that both extracts prolong the induction time at every supersaturation ratio, which means the system needs a longer period to nucleate detectable crystals. Also the absorbance is lower which means the system forms fewer detectable crystals when compared to the baseline (control) experiment where there was no extract.

From Appendix A, it can be seen that both extracts retard nucleation induction time and lower nucleation rate since the maximum increasing slope of absorbance is lower than for the
control. The same trend applies for all supersaturation ratios tested. This observation is in contrast to the hypothesis of the effect of extract calcium concentration.

Also from the light absorbance measurement, the inhibition on nucleation and aggregation can be estimated using method proposed by Hess. The percent of nucleation inhibition can be calculated by:

\[
\% \text{ inhibition of nucleation} = \left[1 - \frac{S_{Nm}}{S_{Nc}}\right] \times 100 \quad (4-1)
\]

\[
\% \text{ inhibition of aggregation} = \left[1 - \frac{S_{Am}}{S_{Ac}}\right] \times 100 \quad (4-2)
\]

\(S_{Nm}\) is the maximum slope of increase of absorbance with time for the experiment with a modulator of interest. \(S_{Nc}\) is the maximum slope of increase in absorbance with time for the control experiment. \(S_{Am}\) is the maximum slope of decrease of absorbance with time for the experiment with a modulator of interest. \(S_{Ac}\) is the maximum slope of decrease of absorbance with time for the control experiment.

Using Equation 4-1, the percent nucleation inhibitory effect of Khella extract is shown in the Figure 4-6.

![Figure 4-6 Nucleation inhibitory effect of both Khella extracts.](image)

66
It is clear that nucleation is greatly inhibited with for all supersaturation studies with the addition of Khella extract. At supersaturation 2.0, the light absorbance curve with the Egyptian Khella extract does not show an increase of slope, therefore, a 100% nucleation inhibition is assumed. Both Khella extracts show more than 50% inhibition of nucleation at every supersaturation. Thus, the addition of both Khella extracts prevents nucleation of calcium oxalate crystal.

As the extracts delayed the absorbance maximum, the data collected do not allow us to draw definite conclusions on their effect on aggregation of calcium oxalate crystals (see Appendix A). However, the data with supersaturation 2.8 do indicate that both extracts may retard aggregation.

From the ICP measurements, it can be concluded that both herb extracts contain some level of calcium, thus the addition of Khella extract into the mixture should have increased the calcium concentration resulting in a higher supersaturation ratio. If the nucleation of calcium oxalate crystals is induced at a higher supersaturated solution, also leading to a decrease in induction time, the nucleation mechanism would be based on supersaturation alone. However, the results show retardation of induction time and inhibition of nucleation so it can be assumed that some constituents in both extracts act as inhibitor of nucleation. This could be done by changing surface energy, complexing free calcium or oxalate ions in the solution, all of which will prevent crystal formation.

**Calcium titration measurement**

A calcium titration study was done to confirm the light absorption measurements. The amount of free calcium in the solution was detected using a calcium electrode. The depletion of calcium concentration indicates that free calcium ions become less available due to precipitation of calcium oxalate crystals.
The titration measurement shows that the addition of Khella (Egyptian) extract maintains the level of free calcium ions in the solution (Figure 4-7). The higher amount of free calcium ions initially with the addition of Khella extract is explained by the ICP results that show that the Khella extract contains calcium. Therefore, some substances in the Khella extracts might compete with oxalate to bind free calcium ions which would lower the precipitation of calcium oxalate crystals and increase the induction time. Also, the titration shows that when mixing the extract with only oxalate solution, there is no calcium depletion as seen in the control.

**Crystal morphology studies**

The crystal morphology of each nucleation experiment was studied using both optical microscope and SEM.

The SEM results show that the crystal structure of the control solution at every supersaturation is of only calcium oxalate monohydrate crystals (Figure 4-8). Interestingly, the crystal morphology from the solution with both extracts contains the calcium oxalate dihydrate form instead of COM crystals (Figures 4-9 and 4-10).
Figure 4-8 SEM picture of COM crystals formed at SS 2.8. A and B are EDS intensity peaks at line A and B on COM crystals.
Figure 4-9 SEM image of the calcium oxalate crystals formed at SS 2.8 with 1 mg/ml of Egyptian Khella extract. A) EDS intensity peak of COD crystal (line A) B) EDS intensity peaks of calcium oxalate crystal at line B.
Figure 4-10  SEM picture of calcium oxalate crystals formed at SS 2.8 with 1 mg/ml of Turkish Khella extract. A) EDS peaks at torus structure (line A). B) EDS peaks at COD structure (line B).
Figure 4-11  SEM picture of calcium oxalate crystals formed at SS 2.4 with 1 mg/ml of Turkish Khella extract.

The calcium oxalate trihydrate structure was not found in any solution. However, the solution with Turkish extract has the torus structure (donut-shape) particle. The torus structure of the higher supersaturations (Figure 4-10) has more rounded edges than those at lower supersaturations (Figure 4-11). It is also seen that the torus structure at low supersaturation (Figure 4-11) has a twinning structure close to the COM crystals. Therefore, it is suspected that the torus structure is the transformation structure from COM crystal. Some components in the Turkish extracts might specifically adsorb onto the COM crystal face and inhibit growth at that surface leading to the rounded structure at higher supersaturation. The change in the calcium oxalate structure nucleated in the presence of the extracts is beneficial to kidney stone treatment.
Wesson and Ward reported that COD structure showed lower adhesion surface force leading to less aggregation of crystal and less crystal adherence to the epithelial cells [64]. Also, the torus structure might be helpful since the particle is relatively small compare to COM and COD and the smooth edge of particle might do less damage to the kidney epithelial cells and thus might be easier to pass through the nephrons. The EDS results on each structure confirmed that all the crystals forms are the components of calcium, oxygen and carbon which are the component of calcium oxalate structure. Other components such as potassium and chloride are also occasionally found due to the contaminations or due to the drying effect of the salts from original solutions of calcium chloride and potassium oxalate.

**Surface energy and Gibbs free energy calculation**

The surface energy and Gibbs free energy can be calculated using the classical homogeneous nucleation theory discussed in Chapter 2. Sohnel and Garside had stated that by plotting \( \log(T_{\text{ind}}) \) against \( 1/(\log SS)^2 \) over a wide range of supersaturation a line with two different slopes will be obtained (Figure 4-13). The slope at high supersaturation ratio is for a homogeneous dominated nucleation. The slope at low supersaturation ratio is for a heterogeneous dominated nucleation.

\[
y = -4.7157x + 8.6197
\]

Figure 4-12 Linear relationship between \( \ln SS \) and \( \ln T_{\text{ind}} \) for the control solution.
Heterogeneous nucleation dominance

Homogeneous nucleation dominance

$y = 1.5568x - 2.4954$

Figure 4-13  Induction time as a function of supersaturation plot used for surface energy calculation.

Because there are time limitations for doing experiments at high supersaturation ratios, the supersaturation ratio range of 2.0 to 2.8 was selected for our experiments. From the experimental data with supersaturation 2.2 to 2.8, a linear relationship between natural log of supersaturation and natural log of induction time is obtained (Figure 4-12). Then this linear equation is used to extrapolate the induction time over a wide range of supersaturation [65, 66]. The slope of a homogeneous dominated nucleation straight line is used to calculate surface energy since our experiments are based on homogeneous nucleation.

SEM pictures show that with the addition of Khella extract, the calcium oxalate crystals have a primary form of calcium oxalate dihydrate. Therefore, when calculating the surface energy for the experiment with the addition of Khella extract, we need to use the constants for COD. Also the amount of calcium ions in the extract is considered to have an effect in changing supersaturation ratios of the system. The new supersaturation ratios are calculated. It is found
that Khella extract lowers supersaturation despite the fact that the extract itself increases the
calcium concentration of the system (Tables 4-3 and 4-4).

These new supersaturation ratios are used in the calculation of surface energy of the
calcium oxalate crystals that formed in the presence of Khella extract. The surface energy values
obtained are shown in Table 4-5.

Table 4-3 New supersaturation ratio calculation of the system with Egyptian extract.

<table>
<thead>
<tr>
<th>SS</th>
<th>Starting Concentration (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>[Ca^{2+}]</td>
</tr>
<tr>
<td></td>
<td>[Ca^{2+}]</td>
</tr>
<tr>
<td></td>
<td>[Ca^{2+}]</td>
</tr>
<tr>
<td>2.0</td>
<td>0.430</td>
</tr>
<tr>
<td>2.2</td>
<td>0.473</td>
</tr>
<tr>
<td>2.4</td>
<td>0.516</td>
</tr>
<tr>
<td>2.6</td>
<td>0.559</td>
</tr>
<tr>
<td>2.8</td>
<td>0.602</td>
</tr>
</tbody>
</table>

Table 4-4 New supersaturation ratio calculation of the system with Turkish extract.

<table>
<thead>
<tr>
<th>SS</th>
<th>Starting Concentration (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>[Ca^{2+}]</td>
</tr>
<tr>
<td></td>
<td>[Ca^{2+}]</td>
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<tr>
<td></td>
<td>[Ca^{2+}]</td>
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<td>2.0</td>
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<tr>
<td>2.2</td>
<td>0.473</td>
</tr>
<tr>
<td>2.4</td>
<td>0.516</td>
</tr>
<tr>
<td>2.6</td>
<td>0.559</td>
</tr>
<tr>
<td>2.8</td>
<td>0.602</td>
</tr>
</tbody>
</table>

Table 4-5 Effect of Khella extract on surface energy of calcium oxalate crystals (*indicating using COD constants)

<table>
<thead>
<tr>
<th>Surface Energy (mJ/m2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
</tr>
<tr>
<td>with Turkish Khella Extract</td>
</tr>
<tr>
<td>with Egyptian Khella Extract</td>
</tr>
</tbody>
</table>

The surface energy calculated for COM in deionized water (control solution) is 19.13
mJ/m². The calculated surface energies in aqueous solution using constant composition methods
by other investigators are approximately 13-29 mJ/m² [67, 68]. When compare our surface
energy with others, the values are relatively close, so the surface energy determination from the induction period in our study is reasonable.

The calculated surface energies are lower for the calcium oxalate crystal nucleated in the system with addition of Khella extract. This implies that calcium oxalate crystals in solution with Khella extract should be easier to form, which is in contrast to the results from the induction times. The surface energy values for the system with Khella were calculated using the COD constants since it is the main crystal morphology shown in SEM pictures. However, considering the standard deviation, the surface energy differences can be considered close together. Haselhuhn et al also compared the surface energy of COM and COD crystals formed at 50 °C. They showed that COD crystal has slightly lower value than that of COM. However, their range of surface energies, 90 mJ/m² for COM and 70 mJ/m² for COD, are much higher than our values since the experiments are done in different temperature, but, it shows the same trends. The COD crystal is known to be a precursor for the COM crystals in biological systems. Therefore, COD crystals are believed to form first then transformed to COM crystals, which should be able to explain the lower surface energies in the presence of Khella extract. However, this is in contrast with the phase stability of calcium oxalate crystals. COM is the most stable form of crystal therefore the surface energy of nuclei should be the lowest of all other forms.

The surface energy is used for calculation of Gibbs free energy of formation. The calculation is based on the homogeneous nucleation assumption, where the value is the summation of a volume term and a surface term as mentioned previously in Chapter 2.

\[ \Delta G_N(r) = \frac{4}{3} \pi r^3 \Delta G_v + 4\pi r^2 \gamma \]  \hspace{1cm} (4-1)

where \( \Delta G_N \) is Gibbs free energy of formation/nucleation, \( \Delta G_v \) is the change in volume free energy, \( r \) is radius of nuclei, and \( \gamma \) is surface energy.
As mentioned before, the critical free energy barrier of formation is the point that the energy from the volume term overcomes the surface term. Therefore, to calculate the critical free energy barrier of formation, $\Delta G_{\text{crit}}$, the derivative of Gibbs free energy of formation with respect to the radius of nuclei, $d(\Delta G_N)/dr$ is set to equal zero and set $r$ to equal critical radius of nuclei, $r_{\text{crit}}$ (Equation 4-2 and 4-3).

$$\frac{d(\Delta G_v)}{dr} = 4\pi r^2 \Delta G_v + 8\pi r \gamma = 0 \quad (4-2)$$

$$4\pi r_{\text{crit}}^2 \Delta G_v = -8\pi r_{\text{crit}} \gamma \quad (4-3)$$

By rearranging the terms, the change of volume free energy, $\Delta G_v$, is equal to

$$\Delta G_v = -\frac{2\gamma}{r_{\text{crit}}} \quad (4-4)$$

Also, $\Delta G_{\text{crit}}$ can be calculated from $\Delta G_N$ at $r$ equals to $r_{\text{crit}}$ as shown below.

$$\Delta G_{\text{crit}} = \Delta G_N (r_{\text{crit}}) \quad (4-5)$$

Substituting $r_{\text{crit}}$ to equation

$$\Delta G_N (r_{\text{crit}}) = \frac{4}{3} \pi r_{\text{crit}}^3 \Delta G_v + 4\pi r_{\text{crit}}^2 \gamma \quad (4-6)$$

Then substituting $\Delta G_v$ from Equation 4-4.

$$\Delta G_N (r_{\text{crit}}) = \frac{4}{3} \pi r_{\text{crit}}^3 (-\frac{2\gamma}{r_{\text{crit}}}) + 4\pi r_{\text{crit}}^2 \gamma = \frac{4}{3} \pi r_{\text{crit}}^2 \gamma \quad (4-7)$$

$$\Delta G_{\text{crit}} = \frac{4}{3} \pi r_{\text{crit}}^2 \gamma \quad (4-8)$$

Also, by rearranging Equation 4-4, the $r_{\text{crit}}$ value can be written in $\Delta G_v$ term.

$$r_{\text{crit}} = -\frac{2\gamma}{\Delta G_v} \quad (4-9)$$

Therefore, by substituting $r_{\text{crit}}$ from Equation 4-8 and rearranging the terms, we obtain the equation of change in volume free energy, $\Delta G_v$, in the term of the critical free energy barrier (Equation 4-10).

$$\Delta G_v = \left(\frac{16}{3} \frac{\pi \gamma^3}{\Delta G_{\text{crit}}}\right)^{\frac{1}{2}} \quad (4-10)$$
Thus, substituting the ΔGv value from Equation 4-10 to Equation 4-1, we get the equation of Gibbs free energy as the function of nuclei radius and ΔGcrit

\[ \Delta G_N (r, \Delta G_{\text{crit}}) = \frac{4}{3} \pi r^3 \left( \frac{16 \pi \gamma^2}{3 \Delta G_{\text{crit}}} \right)^{\frac{1}{2}} + 4 \pi r^2 \gamma \]  

(4-11)

From the Gibbs-Thompson equation, rcrit is obtained in terms of molar volume (\(V_M\)), surface energy (\(\gamma\)), supersaturation ratio (S), gas constant (R), and temperature (T) [69].

\[ \ln S = \frac{2M \gamma}{RT \rho v} = \frac{2V_M \gamma}{RT r_{\text{crit}}} \]  

(4-12)

\[ r_{\text{crit}} = \frac{2V_M \gamma}{RT \ln S} \]  

(4-13)

Therefore, substituting rcrit from Equation 4-13 to Equation 4-8, we get

\[ \Delta G_{\text{crit}} = \frac{4}{3} \pi \left( \frac{2V_M \gamma}{RT \ln S} \right)^2 \gamma = \frac{16 \pi}{3} \left( \frac{V_M^2 \gamma^3}{(RT)^2 (\ln S)^2} \right) \]  

(4-14)

From these derivations, the Gibb free energy of formation can be calculated using the surface energy and supersaturation ratios.

Figure 4-14 shows the decrease in free energy barrier and critical radius of nuclei with the increase of supersaturation ratio. This result confirms the faster induction time of higher supersaturation of calcium oxalate. At high supersaturation, the system requires lower energy and smaller nuclei to precipitate stable crystals. The critical free energy barrier and the critical radius of nuclei are lower at higher supersaturation, leading to faster nucleation rate of calcium oxalate crystals.

The effect of Khella extract on the change in Gibbs free energy of formation was also studied (Figure 4-15 and Appendix B). However, for the systems with extract, the supersaturation is lower (Tables 4-3 and 4-4). It was decided to plot, the change in Gibbs free energy against the initial supersaturation of calcium and oxalate concentration (based on the amount added)
Figure 4-14 Change in Gibbs free energy of formation with respect to supersaturation ratios

From Appendix B, the Gibbs free energy barrier and critical radius of nuclei increases with the addition of Egyptian Khella extract or Turkish Khella extract at every initial supersaturation of calcium and oxalate solution. Both extracts shift the critical nuclei radius to larger values and also increase the free energy barriers of formation. Therefore, the system needs larger nuclei and higher energy to overcome the barrier in order to precipitate stable crystals. These results explain the longer induction time effect of both Khella herbal extracts.

Since COD crystals are less stable than the COM crystals, the higher critical energies barrier and higher critical radius of nuclei explains the observation of COD crystals when Khella extract is added. The phase transformation of COD to COM occurs because the Gibbs free energy of COM crystals is lower as shown in the control solution. When the system reaches
equilibrium, it should go to the lower energy state of COM. However, the crystals observed after 30 minutes of light absorbance measurement remained in dihydrate structures for both Khella extracts. Therefore, some substances or components in the Khella extract might prevent the phase transformation of COD to COM in the systems.

Figure 4-15  Effect of Khella extract on $\Delta G_N$ at initial supersaturation of SS 2.0

The critical energy barrier is also plotted with respect to each system’s actual supersaturation in Figure 4-16. The plot shows that both extracts have no significant change in critical free energy barriers at every supersaturation. Therefore, it can be concluded that the longer induction time effect of Khella extract is based on the lower supersaturation of the system by forming and preserving the calcium oxalate dihydrate crystals.
Cell-retention of calcium oxalate crystal

The interaction force between cell lines, LLC-PK1 and MDCK and COM crystal was studied using AFM. It was found in earlier studies that there is no attraction force between LLC-PK1 cells and a COM crystal attached to the tip [19]; only repulsion was found in both long range and short range for all conditions of LLC-PK1 cells and all solutions used in the experiment. The no adhesion force with the LLC-PK1 cells agrees with the fact that there is a lack of crystal deposition in the proximal tubular section (representation of LLC-PK1 cell line). However, adhesion force is found for the MDCK epithelial cell and a COM crystal on the AFM tip in artificial urine solution medium. This may be due to the different brush border member structure and biochemistry of the cells. Figure 2-4 shows the differences in cell brush border membrane structure. The cell membrane structure of the proximal tubular (LLC-PK1) section
has long and close-packed microvilli, while the cell membrane structure of the collecting duct (MDCK) section has short and smooth microvilli. Also, crystal adhesion to the cells depends on the structure of the brush border membrane, the differences in protein composition of the cells and the flow rate of urine in each section. Therefore, this study is focused more on the adhesion force between COM crystal and MDCK cells.

![Graph showing effect of Khella extract on COM crystals and MDCK interaction force by AFM.](image)

Figure 4-17  Effect of Khella extract on COM crystals and MDCK interaction force by AFM.

To measure the adhesion force between COM crystal and MDCK cells, the force was measured during the retraction of the AFM cantilever out of the surface. Upon approaching the COM crystal on the cantilever tip to the MDCK cells, it causes a push to the cell itself and the repulsion force is caused by the elasticity of the cell. When slowly removing the cantilever from the cells, the repulsion force (positive force) due to the cell elasticity decreases until the curve crosses the x-axis which is the point when the cell returns to its initial stage. Then the negative force is measured if there is adhesion between the COM crystals and kidney epithelial cells.
Figure 4-17 shows no negative interaction force when the Khella extract is present. The error bars indicate standard deviations at the point of maximum adhesion. Some raw AFM measurements are shown in Appendix C. The absence of negative interaction force result implies that there is no adhesion force between COM and MDCK cells. Therefore, Khella extract might have components that prevent the COM crystals from sticking to the MDCK cells. It is reported that the kidney epithelial cells have the affinity to attract COM crystals due to electrostatic attraction between the calcium-rich phase of calcium oxalate crystals and the anionic charge of the phospholipids of the epithelial cell membrane. Khellin and visnagin was reported to have antispasmoic action to smooth muscle cells. Both khellin and visnagin have the calcium channel blocking which prevents calcium ions from permeating through the calcium channel. The calcium ions releasing action of the calcium channel is the signal sent to the smooth muscle cell to retract. When the channel is blocked, the signal from the calcium channel can not be sent so the cell is relaxed. Khella extract, might have the same effect on MDCK cells so MDCK cell interaction to calcium is limited, hence the MDCK cell is relaxed and does not exert adhesion force to COM crystals.

Effect of Khella Extract Components on Calcium Oxalate Crystallization

The previous section shows that the Khella extract has positive results in preventing the kidney stone formation. Addition of Khella extract reduces the supersaturation of the system and lengthens the induction time. Also, the majority of calcium oxalate crystals formed in the presence of Khella extract is in dihydrate form instead of monohydrate form. The Turkish Khella extract also has the torus structure of calcium oxalate crystals which is suspected to be slow growing COM. This result might be due to specific site absorption of some components of the extract. Therefore, the Khella extract components, khellin, visnagin, calcium, and magnesium, needs to be investigated on calcium oxalate crystallization.
Effect of Khellin and Visnagin on Calcium Oxalate Crystallization

Khellin and visnagin are the organic components of the Khella extract identified from the HPLC as well as in the literature. Khellin and visnagin are believed to act as active constituents that effect curing or preventing the reoccurrence of kidney stone.

Nucleation studies

Pure khellin and visnagin were tested using a range of concentration according to the literature. Literature states that khella had approximately 0.3-1.2 wt% of khellin and 0.05-0.3% of visnagin [11]. A statistical design of experiments is used to determine the significant effects of both components. Khellin and visnagin are the factors of the design. Induction time and nucleation inhibition percent are the responses.

Table 4-6  Three level composites design experiment: the effect of khellin and visnagin on light absorbance measurement

<table>
<thead>
<tr>
<th>Std #</th>
<th>Run #</th>
<th>Concentration (mg/ml)</th>
<th>Induction time (s)</th>
<th>% Nucleation Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Khellin</td>
<td>Visnagin</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>5</td>
<td>0</td>
<td>0</td>
<td>126</td>
</tr>
<tr>
<td>2</td>
<td>13</td>
<td>0.015</td>
<td>0</td>
<td>150</td>
</tr>
<tr>
<td>3</td>
<td>9</td>
<td>0.03</td>
<td>0</td>
<td>140</td>
</tr>
<tr>
<td>4</td>
<td>1</td>
<td>0</td>
<td>0.0025</td>
<td>220</td>
</tr>
<tr>
<td>5</td>
<td>12</td>
<td>0.015</td>
<td>0.0025</td>
<td>120</td>
</tr>
<tr>
<td>6</td>
<td>7</td>
<td>0.03</td>
<td>0.0025</td>
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<td>7</td>
<td>4</td>
<td>0</td>
<td>0.005</td>
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<td>0.015</td>
<td>0.005</td>
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<td>9</td>
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<tr>
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<tr>
<td>13</td>
<td>2</td>
<td>0.015</td>
<td>0.0025</td>
<td>150</td>
</tr>
</tbody>
</table>

Three levels were selected for each khellin and visnagin with zero is the lowest concentration and the highest concentration 0.03 mg/ml for khellin and 0.005 mg/ml for visnagin. Three level composites design was selected for this study. Supersaturation 2.4 by adding calcium and oxalate solution was chosen for this experiment. Table 4-6 shows the design
concentration of khellin and visnagin and the responses, induction time and nucleation inhibition percent, for each run.

Figure 4-18 Contour plot from statistical design on % nucleation inhibition of khellin and visnagin.

Using ANOVA to analyze the data set, no statistical significance model could fit the change of induction time for khellin and visnagin. Therefore, both khellin and visnagin have no significant effect in changing the induction time on nucleation of calcium oxalate crystals.

The nucleation inhibition effect is analyzed using 2FI model, where khellin, visnagin, and the interaction between both of the components are studied. The standard run # 4 is blocked due to the lack of fit. ANOVA shows that visnagin and the two interaction terms are significant model terms. The contour plot shows the interaction effect of khellin and visnagin on the nucleation inhibition of calcium oxalate crystals.

The contour plot shows that the highest nucleation inhibition effect is where the system has high khellin and low visnagin where nucleation inhibition has a value approximately 30%. At high khellin and high visnagin, the nucleation inhibition is lowest, -9 %. However, when comparing this result to the effect of Khella extract on nucleation inhibition, we see that the
Khella extract has much higher inhibitory effect on nucleation. Therefore, khellin and visnagin are not the main component that is involved in the nucleation inhibitory effect of Khella extract. This is of course supported by the absence of appreciable amount of khellin and visnagin in the Egyptian Khella used in this study.

Crystal morphology studies

After each light absorbance measurement, the crystals were collected and prepared for SEM in the same manner as done previously. Figure 4-19 shows the SEM and EDS analysis of the crystals collected after run #11.

Figure 4-19 shows the SEM pictures of calcium oxalate crystal with the effect of khellin and visnagin. It is clear that crystals formed were all calcium oxalate monohydrate (COM). Some of the potassium chloride salts crystals are also found due to the contamination mentioned before. These results show that khellin and visnagin do not have any effect on changing crystal morphology from COM to COD or the torus structure.

Figure 4-19 SEM pictures of calcium oxalate crystals formed at SS 2.4 with 0.015 mg/ml Khellin and 0.0025 mg/ml visnagin. A) EDS intensity peaks of COM crystal (line A). B) EDS intensity peaks of KCl crystal (line B).
**Effect of Calcium and Magnesium on Calcium Oxalate Crystallization**

Calcium and magnesium are also known as important factors of calcium oxalate crystallization. The ICP result shows that Khella extract contains both of these ions. Therefore, the effect of calcium and magnesium is investigated in our studies.

*Nucleation studies*

The effect of magnesium was investigated using light absorbance measurements with results shown in Appendix F. The induction time was estimated and is shown in Table 4-7. Also the inhibitory effect of magnesium on calcium oxalate crystal nucleation was estimated (Figure 4-20).

Table 4-7. Effect of 0.1 mM Mg$^{2+}$ on induction time of calcium oxalate nucleation

<table>
<thead>
<tr>
<th>SS</th>
<th>Control (s)</th>
<th>Induction time (s) with 0.1 mM Mg$^{2+}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>376±10</td>
<td>173±24</td>
</tr>
<tr>
<td>2.2</td>
<td>128±43</td>
<td>140±4</td>
</tr>
<tr>
<td>2.6</td>
<td>74±5</td>
<td>60±4</td>
</tr>
<tr>
<td>2.8</td>
<td>38±2</td>
<td>45±8</td>
</tr>
</tbody>
</table>

Table 4-7 shows that magnesium does not have significant effect on induction time retardation. From the induction time obtained, the surface energy of nuclei was calculated to be 18.5 ±0.67 mJ/m$^2$, which is relatively close to the surface energy of the control as shown previously. COM constants were used for the surface energy calculation because from the SEM picture the crystal formed has mainly COM structure (Figure 4-21). The SEM picture clearly shows that the change in crystal morphology seen with the presence of Khella extract does not depend on magnesium level in the extract alone.

The inhibition of calcium oxalate crystal nucleation estimated from light absorbance measurements was also calculated. Magnesium shows inhibitory effect on calcium oxalate nucleation (Figure 4-20). At supersaturation 2.0, the percent nucleation inhibition has a negative value, which means it promotes nucleation. But this might be due to experimental error given the
error bar shown. Nevertheless, the percent of nucleation inhibition from magnesium is less than what we get from Khella extract. Thus it can be concluded that magnesium concentration in the extract does not have a significant contribution on the inhibition effect of calcium oxalate crystallization of the Khella extract.

![Graph](image)

**Figure 4-20**  Inhibitory effect of magnesium on calcium oxalate crystal nucleation.

J. Jeon and H. El-Shall investigated the effect of high calcium ratio, hypercalciuria, previously [39]. It was found that hypercalcuria, has faster induction time, as well as the hyperoxaluria, high oxalate, because the supersaturation is increased.

Also high calcium ratio has a structural modification effect on the calcium oxalate crystals nucleated. The crystals formed with high level of calcium ratios had rounder edges as seen in Figure 4-22. This structure is closer to the bi-pyramid structure of COD than the COM monoclinic structure. However, there was no defined COD structure observed in this high calcium concentration in contrast to what was observed with Khella extracts.
Figure 4-21  SEM picture of calcium oxalate crystals formed at SS 2.6 with the addition of 0.1 mM magnesium.

Figure 4-22  Calcium oxalate crystals in high calcium concentration (taken from Jeon’s thesis) [39].
Several additives are suggested in the literature to induce the COD crystals in *in vitro* crystallization studies. High calcium to oxalate ratio was claimed to be one of preferable conditions for COD [70]. Therefore the structure observed in Figure 4-22 might be calcium oxalate crystals during the transformation of less stable COD to a stable COM structure. To sustain the COD crystal, the system should have other substances that can stabilize the COD phase. Magnesium was suggested to play this stabilizing role for the COD structure [71]. Therefore, the COD crystals formed with the presence of Khella extract might involve the addition of calcium and magnesium from Khella extract. However, it may be an interaction effect of both calcium and magnesium.

**Cell retention studies**

Lieske et al. investigated the effect of various concentrations of calcium and magnesium in Tris Buffer Saline (TBS) solution on the $^{14}$C COM crystals attachment on MDCK cells. Both magnesium and calcium were found to have higher attachment of COM crystals to MDCK cells, hence higher adhesion [53]. AFM measurements in this study were done repeating the conditions of Leiske et al. There is a good correlation between the present AFM results and the results from Leiske et al (Figure 4-23). Both studies show a critical concentration for both Ca$^{2+}$ and Mg$^{2+}$ ions where the maximum adhesion forces appear. Also it was found that the solution with Ca$^{2+}$ ions had much larger adhesion force than the solution with Mg$^{2+}$ ions (Table 4-8).

The AFM results show that the adhesion force first increases then decreases with increasing calcium and magnesium concentration, but is not eliminated. Recall that the AFM study on Khella extract showed no adhesion force at all. Therefore, the presence of calcium and magnesium in Khella extract has no significant contribution on the absence of adhesion force between COM crystal and MDCK cells.
From these studies, it is shown that all the Khella extract components studied so far, khellin, visnagin, calcium, and magnesium, do not contribute to the calcium oxalate crystallization inhibition of the extract. Only magnesium shows minor retardation of induction time and inhibitory effect on calcium oxalate nucleation. None of the components shows the crystal structure altering from COM to COD or the torus structure. Also, adhesion force is shown with calcium and magnesium, while Khella extract prevents adhesion between COM and MDCK cells. Therefore, there should be some other components in Khella extract that contribute to the inhibitory effect of calcium oxalate crystallization and cell-retention.

Table 4-8  Adhesion force between MDCK cells and COM crystal in Ca\textsuperscript{2+} and Mg\textsuperscript{2+} solutions.

<table>
<thead>
<tr>
<th>Concentration of ions (mM)</th>
<th>Fad for Ca\textsuperscript{2+} (nN)</th>
<th>Fad for Mg\textsuperscript{2+} (nN)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.12±0.01</td>
<td>0.12±0.01</td>
</tr>
<tr>
<td>25</td>
<td>0.20±0.02</td>
<td>-</td>
</tr>
<tr>
<td>50</td>
<td>0.38±0.04</td>
<td>0.12±0.01</td>
</tr>
<tr>
<td>100</td>
<td>0.51±0.05</td>
<td>0.20±0.02</td>
</tr>
<tr>
<td>200</td>
<td>0.27±0.03</td>
<td>0.06±0.01</td>
</tr>
<tr>
<td>400</td>
<td>0.26±0.03</td>
<td>-</td>
</tr>
</tbody>
</table>

Figure 4-23  Effect of Ca\textsuperscript{2+} and Mg\textsuperscript{2+} on adhesion force between COM crystal and MDCK cells. Line 1 and 2 are effects of Ca\textsuperscript{2+} and Mg\textsuperscript{2+} respectively on adhesion force measured by AFM. Line 3 and 4 are effects of Ca\textsuperscript{2+} and Mg\textsuperscript{2+} respectively on adhesion force measured by Lieske.
Effect of Other Urinary Species on Calcium Oxalate Crystallization

Other urinary species such as citrate, oxalate, urinary proteins, and cellular membrane debris were also studied on their effect on crystallization of calcium oxalate. Some species are known to be an inhibitor of kidney stone formation, such as citrate and albumin proteins. Others, oxalate and cellular membrane debris, are known as promoters.

Effect of Citrate on Calcium Oxalate Crystallization

Citrate is a known inhibitor of calcium oxalate crystal nucleation due to the anionic charge of citrate structure. It has been studied thoroughly on its inhibition of calcium oxalate crystallization [7, 30, 38, 47, 70, 72]. Citrate was investigated here to compare its effects to those of the Khella extracts.

Nucleation studies

The UV-Vis absorbance graph confirms that citrate prevents nucleation by increasing induction time at every supersaturation ratio (Table 4-9, Appendix G, and Figure 4-24). An increase in induction time with the presence of citrate is due to the change in supersaturation ratio. When citrate is introduced to the system, the supersaturation ratio decreases because of the formation of a soluble calcium citrate complex as discussed before in chapter 2. Such a complex makes the calcium ions in the system less available to interact with oxalate and form calcium oxalate crystal.

Also, the percent nucleation inhibition of citrate can be estimated from the light absorbance measurements. It was found that citrate also inhibited the nucleation rate. However, when compared to the Khella extract, citrate has less nucleation inhibition effect as (Figure 4-25). Also at supersaturation 2.8, citrate loses its nucleation inhibitory effect.

The result from the SEM picture (Figure 4-26) shows that the calcium oxalate crystals formed in the presence of citrate have a flatter and less sharp edges structure of calcium oxalate
monohydrate. Citrate is known to specifically bind on the surface of COM crystals to minimize configuration energy.

Figure 4-24  Effect of citrate on light absorbance measurement at supersaturation 2.0

Table 4-9  The induction time estimation from light absorbance measurement with the presence of citrate.

<table>
<thead>
<tr>
<th>SS</th>
<th>Control</th>
<th>Induction time (s) with Citrate</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.0</td>
<td>376±10</td>
<td>425±17</td>
</tr>
<tr>
<td>2.2</td>
<td>128±43</td>
<td>290±36</td>
</tr>
<tr>
<td>2.6</td>
<td>74±5</td>
<td>131±9</td>
</tr>
<tr>
<td>2.8</td>
<td>38±2</td>
<td>83±2</td>
</tr>
</tbody>
</table>
Figure 4-25  Inhibitory effect of 0.1mM citrate on calcium oxalate nucleation.

Figure 4-26  The effect of citrate on calcium oxalate monohydrate crystal structure.
The EDS spectrometry on a single crystal was performed. The result shows the peak of calcium, carbon and oxygen, which confirmed that these crystals were calcium oxalate. The gold peaks are also shown due to the gold coating of the sample.

Qiu et al. reported that citrate preferable binds to a (-101) step more than a (010) face, which causes the calcium oxalate monohydrate crystal to have smoother and rounder edges [72]. However, the calcium oxalate monohydrate structure modification from citrate does not show the torus structure that was seen with Turkish Khella. Some substances present in Khella extract might act as citrate does on the crystal morphology. Some components with anionic charges interact with calcium and also specifically bind to the calcium rich surface. This specific binding inhibits the growth of specific faces and induces the smooth and rounded edges like the torus structure.

The calculated surface energy of the system with the addition of 0.1 mM citrate is 19.5±0.2 mJ/m². When compared to the surface energy of the control system calculated earlier which is 19.13±1.19 mJ/m², it can be concluded that citrate does not have appreciable effect in changing the surface energy of nuclei, even though it changes the morphology.

The critical free energy barrier at each supersaturation is calculated and plotted as seen in Figure 4-28. It is seen that citrate does not change the critical free energy barrier of the system at
every supersaturation. Therefore, it can be concluded that even though citrate increases induction time and changes crystal morphology, it does not change the free energy of the system. So the crystal nucleates should still be COM in structure. The change in crystal morphology by citrate should be due to slower crystal growth at specific sites of COM crystals as discussed before.

![Graph showing the effect of citrate on change in critical free energy barrier](image)

**Figure 4-28** Effect of citrate on change in critical free energy barrier.

**Cell-retention studies**

Besides the effect of citrate on nucleation of calcium oxalate crystals, cell-retention was also studied. Figure 4-29 shows the plot of interaction force between COM crystal and MDCK cells as a function of indentation. It is seen that the presence of citrate in AUIS solution medium reduces the adhesion forces between COM and MDCK cells. The adhesion force in the control (AUIS) solution is approximately equal to -0.4 nN and equal to -0.25 nM in the AUIS containing 0.1 mM citrate solution. The differences in indentation distance at maximum adhesion
force depend on the thickness of the cells at the measuring point where the COM crystal on cantilever tip touches the cells.

![Graph showing interaction force measurement between COM crystal and MDCK cells in control and with 0.1 mM of citrate in AUIS solution.](image)

Figure 4-29 The interaction force measurement between COM crystal and MDCK cells in control (AUIS) and with 0.1 mM of citrate in AUIS solution.

**Effect of Cellular Membrane Debris on Calcium Oxalate Crystallization**

When the renal epithelial cells are injured, the degradation of epithelial cells occurs. The membrane debris in the urine is believed to have a role in kidney stone formation because of its presence in the organic matrix of the kidney stone.

Debris from MDCK and LLC-PK1 kidney epithelial cell membrane was added to the calcium and oxalate solution at various supersaturation ratios. The data showed that the debris
from both cell lines enhanced the nucleation of calcium oxalate crystal by decreasing induction time at low supersaturation (Figure 4-30 and Table 4-10).

![Graph showing the effect of cellular membrane debris on light absorbance measurement at SS 2.0](image)

**Figure 4-30** Effect of cellular membrane debris (MDCK and LLC-PK1) on light absorbance measurement at SS 2.0

**Table 4-10** Effect of cellular membrane debris on the induction time at various supersaturation

<table>
<thead>
<tr>
<th>SS</th>
<th>Control</th>
<th>LLC-PK1</th>
<th>MDCK</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>163</td>
<td>140</td>
<td>100</td>
</tr>
<tr>
<td>2.2</td>
<td>90</td>
<td>90</td>
<td>90</td>
</tr>
<tr>
<td>2.4</td>
<td>42</td>
<td>65</td>
<td>60</td>
</tr>
<tr>
<td>2.8</td>
<td>31</td>
<td>40</td>
<td>35</td>
</tr>
<tr>
<td>3.0</td>
<td>25</td>
<td>20</td>
<td>20</td>
</tr>
<tr>
<td>3.2</td>
<td>18</td>
<td>15</td>
<td>11</td>
</tr>
</tbody>
</table>

By providing nucleation sites, cellular membrane debris increases the nucleation rate. Therefore, the presence of cell membrane debris reduces a free energy barrier for nucleation.

However, when considering that the standard deviation on induction time ranges from 10
seconds to 60 seconds in previous experiments, it can not be concluded that the presence of cellular membrane debris significantly changes it.

Figure 4-31 Effect of cellular membrane debris on nucleation rate inhibition.

The effect of the cellular debris on calcium oxalate nucleation rate is also investigated (Figure 4-31). It shows that the presence of both LLC-PK1 and MDCK cell debris, promotes nucleation of the calcium oxalate crystal. The negative value of percent of nucleation inhibition implies that the system has faster nucleation rate due to the higher maximum increased slope of light absorbance with time. Despite, the small changes in induction period, it is clear that cellular membrane debris induces nucleation by increasing the nucleation rate. The positive percent of nucleation inhibition at supersaturation 2.8 might be due to experimental errors at that supersaturation.
Also, from Figure 4-31, it is seen that the cellular debris from MDCK cell has higher inhibitory effect in most of supersaturations. Therefore, the MDCK cells might have higher affinity to interact with calcium oxalate crystals than LLC-PK1 cells, something consistent with the fact that kidney stones are found more at the collecting duct section (represented by MDCK cells) than in other sections.

The surface energy and Gibbs free energy with the presence cellular membrane debris are calculated and the results are shown below.

Table 4-11 The effect of cellular membrane debris on surface energy of nuclei

<table>
<thead>
<tr>
<th></th>
<th>Surface Energy (mJ/m²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>19.28</td>
</tr>
<tr>
<td>MDCK</td>
<td>20.19</td>
</tr>
<tr>
<td>LLC-PK1</td>
<td>19.65</td>
</tr>
</tbody>
</table>

Figure 4-32 Effect of cellular membrane debris on change in Gibbs free energy of formation at SS 2.2
Table 4-11 shows no significant change in surface energy with the presence of cellular membrane debris. No change in surface energy implies that the cellular membrane debris does not alter the calcium oxalate crystal structure or bind with the crystal surface.

![Figure 4-33 Change in critical free energy barrier as a function of supersaturation with the presence of cellular membrane debris](image)

It is shown in Figures 4-32 and 4-33 that the Gibbs free energy of formation and a critical free energy barrier with the presence of both cellular membranes is slightly higher than for the control. The critical radius of nuclei remains relatively close for all conditions. These results show that the presence of cell debris does not change the free energy of formation which is in contrast to the faster nucleation rate found previously. However, all the surface energy, Gibbs free energy of formation, and critical free energy barrier values are calculated based on the homogeneous nucleation assumption. As discussed previously, the presence of cellular membrane provides the physical site for nucleation to occur. Therefore the system with cellular
debris is subjected to heterogeneous nucleation. So the assumption of homogeneous nucleation with a system that contains cell debris is not valid.

**Effect of Urinary Proteins on Calcium Oxalate Crystallization**

Proteins are another important urinary species that may effect crystallization of calcium oxalate. Albumin was selected to be representative of urinary proteins in this study. Albumin is the most common and most abundant protein in the biological system. Also albumin is found in the organic matrix of kidney stones; therefore, it should be involved in the formation mechanism of kidney stones.

**Nucleation studies**

The light absorbance measurements were performed in the mixture of calcium and oxalate solution at various supersaturation ratios. To study the effect of urinary proteins, the concentration of 15 µg/ml of albumin was selected. The induction time estimation from light absorbance is shown in Table 4-12.
The presence albumin slightly prolongs induction time at every supersaturation when compared with the control experiments. However, this retardation of induction time is not significant when considering the standard deviation. The calculation of surface energy and Gibbs free energy barriers using homogeneous nucleation cannot be assumed in this case. The addition of albumin in the solution provides the foreign body to the system; therefore, our nucleation system with albumin is heterogeneous nucleation. As shown in the result of nucleation study with the presence of cellular membrane debris, the homogenous assumption is not valid with the heterogeneous nucleation.

**Table 4-12** Effect of albumin protein on induction time at various supersaturations

<table>
<thead>
<tr>
<th>SS</th>
<th>Control</th>
<th>With Albumin (15 µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>172±9</td>
<td>177±40</td>
</tr>
<tr>
<td>2.2</td>
<td>105±15</td>
<td>135±9</td>
</tr>
<tr>
<td>2.4</td>
<td>56±14</td>
<td>90±9</td>
</tr>
<tr>
<td>2.8</td>
<td>31±1</td>
<td>40±5</td>
</tr>
</tbody>
</table>

Figure 4-35  Effect of albumin on percent inhibition of calcium oxalate nucleation.
The change of slope in light absorbance measurement is noticed with the addition of albumin protein. The inhibition of albumin protein on calcium oxalate crystals nucleation was estimated using the same method as before. In addition of nucleation inhibition effect of albumin, the effect on calcium oxalate aggregation was also noticed. Unlike all other studies mentioned earlier in this thesis, these experiment sets with albumin show significant decreases in slope after the absorbance reaches maximum value (see Appendix J). The inhibitory effect of albumin on aggregation can be estimated using Equation 4-2. The inhibitory effects of albumin on nucleation and aggregation are shown in Figures 4-35 and 4-36.

![Figure 4-36](image)

Figure 4-36  Inhibitory effect of albumin on calcium oxalate crystals aggregation rate.

Figure 4-35 shows that albumin promotes nucleation at low supersaturation and inhibits nucleation at higher supersaturation. Comparing to the previous results, from Khella extract that have nucleation inhibitory effect in the range of 70% or more, the inhibition effect of albumin protein on nucleation is small.
It is clear that albumin promotes aggregation of calcium oxalate crystals at every supersaturation. Albumin might enhance the aggregation of crystals due to steric attraction by forming polymer bridging between crystals. Our results are in contrast with other previous study which claimed that albumin has a dispersive effect between calcium oxalate crystals by promoting nucleation of smaller calcium oxalate crystals [73].

Kulaksizoglu et al. also suggested the promotion of calcium oxalate nucleation as the concentration of albumin increases [74]. Atmani et al. found that albumin is present in the matrix of all type of stones [29, 75]. The presence of albumin in the stone matrix indicates that albumin should be involved in the calcium oxalate crystals aggregation mechanism. Albumin has anionic molecular structure which has high affinity to calcium [34] which could explain the retardation effect of induction time. Therefore, it is suggested that albumin might bind with the calcium rich phase of calcium oxalate crystals [76]. Since our experiments were done in an unstirred system, the decreased slope of light absorbance is dependent on the crystal settling rate.

The settling rate depends on several factors such as the aggregation and growth of the calcium oxalate crystals in the system. When the crystals are aggregated or grow to a larger size, they will settle down faster due to the heavier weight. Albumin is also a macromolecule with molecular weight approximately 67 kDa; therefore it is possible to settle down after a period of time. Thus, when albumin binds with calcium oxalate crystals, the settling down effect of albumin will also influence the settling down rate of calcium oxalate crystals as well.

Interestingly, the COD crystals structure is also found with the presence of albumin [34]. The COD structure is also found in the presence of Khella extract in our experiments. Therefore, Khella extract might also have an organic macromolecule that has anionic charge like albumin but has smaller molecular weight so it is not settling down in aqueous solution.
Cell retention studies

From the interaction force measurement between a COM crystal and MDCK cells, the experiment in AUIS solution with 5 µg/ml of albumin did not show significant changes in maximum adhesion force (Figure 4-37).

![Graph showing the effect of albumin on interaction force between COM crystal and MDCK cells.](image)

**Figure 4-37** The effect of albumin on interaction force between COM crystal and MDCK cells.

The combination effect of albumin and oxalate was also measured. Three levels of oxalate and albumin were used to investigate the interaction effect between species (Table 4-14).

<table>
<thead>
<tr>
<th>Solution</th>
<th>Albumin (ppm)</th>
<th>Oxalate (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>5</td>
<td>0.1</td>
</tr>
<tr>
<td>2</td>
<td>5</td>
<td>10</td>
</tr>
<tr>
<td>3</td>
<td>15</td>
<td>0.1</td>
</tr>
<tr>
<td>4</td>
<td>15</td>
<td>10</td>
</tr>
<tr>
<td>5</td>
<td>10</td>
<td>5</td>
</tr>
</tbody>
</table>
A shorter range of indentation distance was measured in this experiment. It is suspected that the MDCK cells used had lost their epithelial membrane structure and were left with only basement membrane on the substrate. Oxalate is known to be one of the key components that cause the kidney epithelial cell to rupture. When injured or killed, the cells lose their polarity which leads to membrane degradation and exposition of basement membrane. Therefore, the oxalate content of the solution may have injured the MDCK cells. If basement membrane was present on the substrate this would reduce the indentation distance.

Figure 4-38 shows that at higher concentrations of albumin, the short range repulsive forces increased (line 3 and 4). This increase of repulsive forces might be due to the steric
repulsion from albumin. Albumin protein could be considered as a polymer molecule. At higher concentration, albumin might act as a polymeric stabilizer between the COM crystal and the MDCK cells. The protein might adsorb on the MDCK cell membrane and form a mushroom or packed brush boarder region on the surface, thus causing steric repulsion. Higher oxalate concentration reduces the repulsive force between COM crystal and MDCK cells, due to more injury of the cell from oxalate. When the cell is injured, the cell exposes the anionic-charged phospholipids from the inside layer of the membrane. The presence of anionic phospholipids at the site of cell injury attracts COM crystals by electrostatic force.
CHAPTER 5
CONCLUSION AND FUTURE WORK

Conclusion

In this study, the effect of Khella (Ammi visnaga) on calcium oxalate crystallization was investigated. The light absorbance graph was used to estimate the induction time of nucleated crystals in calcium and oxalate solutions with the addition of extract and other species of interest. Addition of Khella extract, significantly prolongs induction time of nucleation which implies that the system requires longer time to form detectable crystals. Also, the inhibition of nucleation of the extract was studied using the maximum increasing slope of the light absorbance measurements. It showed that both Khella extracts studied, Egyptian and Turkish, succeeded in inhibiting nucleation with an average value of 75%. Thus, besides reducing the pain of the trapped stones by relaxing muscles, Khella extract hinders the nucleation of calcium oxalate crystals, the main crystalline components of the stones.

Crystals of the calcium oxalate crystals formed with the presence of extract are mostly in the COD form and some other less sharp-edged crystalline form of calcium oxalate. The crystals formed in the control solution, a mixture of calcium and oxalate solution alone, were purely well-structured COM crystals. This change in crystal hydrate structure from COM to COD from the addition of Khella extract is also beneficial to the kidney stone formers. COD crystals have higher solubility and thermodynamically less stable. The COD structure of calcium oxalate crystal is also believed to dissolve easier than COM crystals. Also, with smoother edge, the COD crystals, cause less physical damage to the kidney epithelial cells.

Khella extract also shows that it has a prevention effect on the COM crystal adhesion to MDCK kidney epithelial cells. Hence, Khella extract is effective in kidney stone prevention by
easing down the trapped stone through the renal tubule, slows down nucleation of the main crystals of the stone, and reduces the cell adhesion of calcium oxalate monohydrate crystals.

Khella extract components were identified using several techniques. Our study finds that Khella extract received from Turkey has khellin and visnagin as stated in the literature. However, the Egyptian Khella extract does not show appreciable khellin and visnagin in both TLC and HPLC results. This absence of khellin and visnagin in Egyptian Khella extract might be a result from poor storage of the dried seed. Khellin and visnagin might degrade due to humidity, temperature gradient and light exposure. The pure standards of khellin and visnagin were tested on nucleation studies. Using statistical design of experiments, it was shown that both khellin and visnagin have no statistically significant effect on the induction time and percent of nucleation inhibition. The crystals that are formed with the presence of khellin and visnagin are clearly of COM structure and show no sign of other hydrate forms. However, both extracts, Egyptian and Turkish, are found to have an effect, altering morphology and hindering nucleation. Interestingly, the HPLC results show a matching unknown peak for both extracts at a retention time of approximately 19 minutes. This organic component might be the cause for preventive action on hindering stone formation of Khella extract. This unknown peak should be separated using an HPLC separation column, tested on its nucleation effect, and then identified.

Other inorganic components that are suspected to have an influence on calcium oxalate crystallization were investigated. Our results show that Khella extract also contained calcium, magnesium and potassium ions. Calcium ion is one of the main components for nucleating calcium oxalate crystals. Therefore, addition of calcium ion from extract should promote nucleation of calcium oxalate crystals by increasing supersaturation. Our group’s previous studies, Jeon’s thesis, show that the high calcium ratio induces nucleation, or has faster induction
time. However, the calcium oxalate crystal morphology with high calcium ratio is altered to smoother rounder edges as seen in Figure 4.15. There was, however, no evidence of COD crystals and it was concluded that the high calcium ratio does not contribute to the presence of COD crystals in solutions with extract. The effect of magnesium on the nucleation studies was also investigated. Magnesium has no significant effect on retardation of calcium oxalate nucleation and crystal morphology.

The direct force measurement of COM crystal and MDCK cell interaction by AFM shows adhesion force with the presence of both calcium and magnesium in TBS solution. In contrast, the addition of extract shows no adhesion force at all. Therefore, the effect of Khella on cell retention by preventing adhesion between COM and kidney epithelial cells is not influenced from the content of calcium and magnesium in the extract. The extract organic components, such as khellin and visnagin or the unknown components, might prevent the COM crystals from adhering to the kidney epithelial cell.

Citrate is also a known calcium oxalate nucleation inhibitor. The induction time experiments in our study confirmed that citrate retards induction time at every supersaturation. Citrate is an anionic compound which competitively binds calcium into a complex solution. However, citrate does not nucleate COD crystals. The crystals of calcium oxalate formed with citrate were shown to have thinner and rounder edges of COM. But, they were not COD.

The adhesion force measurement in the presence of citrate shows a lower adhesion force between COM and MDCK cells. However, the change is small and the adhesion force still exists unlike what we saw with Khella extract.

Cellular membrane debris was also studied for its effect on nucleation of calcium oxalate crystals. The presence of cellular membrane debris induces nucleation by changing the
nucleating system from homogeneous to heterogeneous. Using the slope of light absorbance, it was shown that the addition of cell membrane debris promotes calcium oxalate nucleation. By providing a site for nucleation, the system requires less energy to precipitate crystals. However, the assumption using classical homogeneous nucleation is not valid in this system since values using homogeneous nucleation theory do not provide a good correlation with the data from the experiments. The system with cellular membrane debris should have lower surface energy, less critical energy barriers than the system without the membrane. However, the results show no change in surface energy and higher critical energy barrier at every supersaturation.

Albumin was used to test the effect of urinary proteins on calcium oxalate nucleation. It was found that albumin inhibits calcium oxalate nucleation by both increasing induction time and percent nucleation inhibition. However, this effect is insignificant when considering the error bar and comparing to the results from Khella extract. Although, albumin is found to have no significant effect on nucleation of calcium oxalate crystals, it is found to promote crystal aggregation.

The aggregation of calcium oxalate crystals from albumin is due to protein binding to the calcium rich phase of crystals and to the settling down effect of the protein itself. From the cell retention studies using AFM, it was shown that there is no change in adhesion force with addition of albumin on the long range forces. However, when measuring the adhesion force with a mixture of albumin and oxalate, a short range force was measured due to MDCK cell losing its epithelial membrane from exposure to oxalate. The short range force represented the interaction between COM crystal and the basement membrane of the MDCK cell. It was found that the high albumin level provides higher repulsive force between COM crystal and the basement membrane. This increasing repulsion could be due to steric inhibition.
**Future Work**

Here are some suggestions for future work:

- Test the effect of pure khellin and visnagin on the AFM force measurements.
- Test the effect of extract and other components on crystal-cell adhesion using the $^{14}$C COM crystals attachment assay.
- With AFM, perform the elasticity measurement of the cell with and without extract.
- Verify crystal structure and do the quantitative analysis of each structure formed using XRD and FTIR.
- Determine the crystal size distribution of the crystal formed using Coulter particle size analysis.
- Study the effect of extract on calcium oxalate nucleation in artificial and human urine environment.
- Study the effect of Khella extract on calcium oxalate crystal aggregation using AFM to measure crystal-crystal interaction force.

But, of course, the major objective of future work should be to:

- Separate the other unknown organic components using HPLC and identify the unknowns.
- Test the effect of unknown organic components on nucleation and cell retention.
APPENDIX A
EFFECT OF KHELLA EXTRACT ON LIGHT ABSORBANCE MEASUREMENT

Figure A-1: Light absorbance measurement with the presence of Khella extract at supersaturation 2.0

Figure A-2: Light absorbance measurement with the presence of Khella extract at supersaturation 2.2
Figure A-3 Light absorbance measurement with the presence of Khella extract at supersaturation 2.4

Figure A-4 Light absorbance measurement with the presence of Khella extract at supersaturation 2.6
Figure A-5 Light absorbance measurement with the presence of Khella extract at supersaturation 2.8
APPENDIX B
EFFECT OF KHELLA ON THE CHANGE IN GIBBS FREE ENERGY OF FORMATION

Figure B-1 Effect of Khella extract on $\Delta G_N$ at initial supersaturation of SS 2.0

Figure B-2 Effect of Khella extract on $\Delta G_N$ at initial supersaturation of SS 2.2
Figure B-3 Effect of Khella extract on $\Delta G_N$ at initial supersaturation of SS 2.4

Figure B-4 Effect of Khella extract on $\Delta G_N$ at initial supersaturation of SS 2.6
Figure B-5 Effect of Khella extract on $\Delta G_N$ at initial supersaturation of SS 2.8
APPENDIX C
AFM MEASUREMENTS OF COM CRYSTAL AND MDCK CELLS INTERACTION FORCE

Figure C-1 Example of AFM measurements of COM-MDCK cells interaction in artificial urine solution with 1 mg/ml of Khella extract.

Figure C-2 Example of AFM measurements of COM-MDCK cells interaction in artificial urine solution.
APPENDIX D
EFFECT OF KHELLIN AND VISNAGIN PURE COMPOUNDS ON LIGHT ABSORBANCE MEASUREMENT

Figure D-1 Light absorbance measurement of standard # 5, 10, 11, 12, 13. (0.015 mg/ml Khellin and 0.0025 mg/ml visnagin)

Figure D-2 Effect of khellin on light absorbance measurement (standard # 1, 2, and 3).
Figure D-3 Effect of visnagin on light absorbance measurement (standard # 5, 1, and 4).

Figure D-4 Comparison of light absorbance measurement of all standard run number.
APPENDIX E
ANOVA FOR % NUCLEATION INHIBITION RESPONSE SURFACE OF KHELLIN AND VISNAGIN PURE COMPOUND

A = Khellin
B = Visnagin

**Response:** % Nucleation Inhibition

**ANOVA for Response Surface 2FI Model**

**Table E-1 Analysis of variance table [Partial sum of squares]**

<table>
<thead>
<tr>
<th>Source</th>
<th>Sum of Squares</th>
<th>DF</th>
<th>Mean Square</th>
<th>F Value</th>
<th>Prob &gt; F</th>
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<tbody>
<tr>
<td>Model</td>
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<td>3</td>
<td>717.1</td>
<td>5.39</td>
<td>0.0253</td>
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<tr>
<td>A</td>
<td>62.29</td>
<td>1</td>
<td>62.29</td>
<td>0.47</td>
<td>0.513</td>
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<tr>
<td>B</td>
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<td>864</td>
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<td>0.0342</td>
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<tr>
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<td>1225</td>
<td>9.21</td>
<td>0.0162</td>
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<tr>
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<td>8</td>
<td>132.96</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lack of Fit</td>
<td>780.51</td>
<td>4</td>
<td>195.13</td>
<td>2.76</td>
<td>0.1749</td>
</tr>
<tr>
<td>Pure Error</td>
<td>283.2</td>
<td>4</td>
<td>70.8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cor Total</td>
<td>3215</td>
<td>11</td>
<td></td>
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</table>

The Model F-value of 5.39 implies the model is significant. There is only a 2.53% chance that a "Model F-Value" this large could occur due to noise. Values of "Prob > F" less than 0.0500 indicate model terms are significant. In this case B, AB are significant model terms. Values greater than 0.1000 indicate the model terms are not significant. If there are many insignificant model terms (not counting those required to support hierarchy), model reduction may improve your model.

The "Lack of Fit F-value" of 2.76 implies the Lack of Fit is not significant relative to the pure error. There is a 17.49% chance that a "Lack of Fit F-value" this large could occur due to noise. Non-significant lack of fit is good -- we want the model to fit.

**Table E-2 Statistic results**

| Std. Dev.  | R-Squared | 0.6691 |
| Mean       | Adj R-Squared | 0.5451 |
| C.V.       | Pred R-Squared | 0.1747 |
| PRESS      | Adeq Precision | 8.862 |

The "Pred R-Squared" of 0.1747 is not as close to the "Adj R-Squared" of 0.5451 as one might normally expect. This may indicate a large block effect or a possible problem with your model and/or data. Things to consider are model reduction, response tranformation, outliers, etc. "Adeq Precision" measures the signal to noise ratio. A ratio greater than 4 is desirable. Your ratio of 8.862 indicates an adequate signal. This model can be used to navigate the design space.
Final Equation in Terms of Coded Factors:
Nucleation Inhibition  =  
+7.20  
+3.56  * A  
-12.00 * B  
-17.50 * A * B

Final Equation in Terms of Actual Factors:
Nucleation Inhibition  =  
-1.85593  
+1403.95480  * Khellin  
+2200.00000  * Visnagin  
-4.66667E+005  * Khellin * Visnagin

Table E-3 Diagnostics case statistics

<table>
<thead>
<tr>
<th>Standard Order</th>
<th>Actual Value</th>
<th>Predicted Value</th>
<th>Residual</th>
<th>Leverage</th>
<th>Student Residual</th>
<th>Cook's Distance</th>
<th>Outlier t</th>
<th>Run Order</th>
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</table>

# Obs with Leverage > 2.00 *(average leverage)

Proceed to Diagnostic Plots (the next icon in progression). Be sure to look at the:
1) Normal probability plot of the studentized residuals to check for normality of residuals.
2) Studentized residuals versus predicted values to check for constant error.
3) Outlier t versus run order to look for outliers, i.e., influential values.
4) Box-Cox plot for power transformations.

If all the model statistics and diagnostic plots are OK, finish up with the Model Graphs icon.
Figure F-1 Effect of magnesium on light absorbance measurement at supersaturation 2.0

Figure F-2 Effect of magnesium on light absorbance measurement at supersaturation 2.2
Figure F-3 Effect of magnesium on light absorbance measurement at supersaturation 2.6

Figure F-4 Effect of magnesium on light absorbance measurement at supersaturation 2.8
APPENDIX G

EFFECT OF CITRATE ON LIGHT ABSORBANCE MEASUREMENT

Figure G-1 Effect of citrate on light absorbance measurement at supersaturation 2.0

Figure G-2 Effect of citrate on light absorbance measurement at supersaturation 2.2
Figure G-3 Effect of citrate on light absorbance measurement at supersaturation 2.6

Figure G-4 Effect of citrate on light absorbance measurement at supersaturation 2.8
APPENDIX H
EFFECT OF CELLULAR MEMBRANE DEBRIS ON LIGHT ABSORBANCE MEASUREMENT

Figure H-1 Effect of cellular membrane debris on light absorbance measurement at supersaturation 2.0

Figure H-2 Effect of cellular membrane debris on light absorbance measurement at supersaturation 2.2
Figure H-3  Effect of cellular membrane debris on light absorbance measurement at supersaturation 2.4

Figure H-4  Effect of cellular membrane debris on light absorbance measurement at supersaturation 2.8
Figure H-5 Effect of cellular membrane debris on light absorbance measurement at supersaturation 3.0

Figure H-6 Effect of cellular membrane debris on light absorbance measurement at supersaturation 3.2
APPENDIX I
EFFECT OF CELLULAR MEMBRANE DEBRIS ON CHANGE IN GIBBS FREE ENERGY OF FORMATION

Figure I-1 Effect of cellular membrane on the change in Gibbs free energy of formation at supersaturation 2.0

Figure I-2 Effect of cellular membrane debris on change in Gibbs free energy of formation at supersaturation 2.2
Figure I-3 Effect of cellular membrane debris on change in Gibbs free energy of formation at supersaturation 2.4

Figure I-4 Effect of cellular membrane debris on change in Gibbs free energy of formation at supersaturation 2.6
Figure I-5  Effect of cellular membrane debris on change in Gibbs free energy of formation at supersaturation 2.8
APPENDIX J
EFFECT OF ALBUMIN PROTEINS ON LIGHT ABSORBANCE MEASUREMENT

Figure J-1 Light absorbance measurement with the effect of albumin protein at supersaturation 2.0.

Figure J-2 Light absorbance measurement with the effect of albumin protein at supersaturation 2.2.
Figure J-3  Light absorbance measurement with the effect of albumin protein at supersaturation 2.4.

Figure J-4  Light absorbance measurement with the effect of albumin protein at supersaturation 2.8.
LIST OF REFERENCES


Saijit Daosukho was born in Bangkok, Thailand. She attended Kasetsart University Laboratory School from grades 1 to 12. Saijit received a scholarship from the Thai Government after graduating from high school to study in particle engineering. She spent one year as a post graduate student at Wyoming Seminary College Preparation School in Kingston, Pennsylvania. After which, she attended Carnegie Mellon University in Pittsburgh, Pennsylvania and obtained her B.S. in materials science and engineering with minor in electronic materials in August 2001.

She began her graduate studies in materials science and engineering department at University of Florida and received her Master of Science in December, 2003. There, she started researching particle interaction and became involved in a kidney stone project under supervisory of Dr. Hassan El-Shall. After completing her Master’s degree, Saijit transferred to chemical engineering department to pursue her Doctor of Philosophy degree in 2004. However, she continued working on her kidney stone project under co-supervision of Dr. Spyros Svoronos and Dr. Hassan El-Shall.

Once she graduates from the University of Florida in December 2007, she will begin work as a research scientist for the Science, Technology, and Environmental Ministry of Thailand.