INVESTIGATION OF THE *IN VIVO* AND *IN VITRO* EFFECTS OF SOME HERBAL PREPARATIONS ON RISK FACTORS FOR CALCIUM OXALATE KIDNEY STONE DISEASE

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yad yad ācarati śresthas

tat tad evetaro janah

sa yat pramānam kurute

lokas tad anuvartate

Whatever action a great man performs, common men follow and whatever standards he sets by exemplary acts, all the world pursues

Bhagavad-gita 3.21

AC Bhaktivedanta Swami Prabhupada

Dedicated to the greatest man I know... KKS

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Investigation of the effects of a Chinese herbal preparation on calcium oxalate crystallization in synthetic urine

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The effect of Folium pyrrosiae (Shi Wei) on calcium oxalate kidney stone risk factors: an in vivo study on South African males

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ABSTRACT

INVESTIGATION OF THE *IN VITRO* AND *IN VIVO* EFFECTS OF SOME HERBAL PREPARATIONS ON RISK FACTORS FOR CALCIUM OXALATE KIDNEY STONE DISEASE

Ronica Ramsout

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Several herbal preparations (*Folium pyrrosiae*, *Desmodium styracifolium*, *Hylocereus trigonus*, *Phyllanthus niruri*, *Orthosiphon stamineus* and Cystone®) were investigated as potential therapeutic and prophylactic agents for kidney stone disease. These studies were executed in the context of the existence of a virtually stone-free (black) and a stone-prone (white) population group in South Africa, with a view of establishing whether their respective renal responses are different.

The independent *in vitro* effects of six plant extracts were tested on the crystallization characteristics of calcium oxalate (CaOx), the predominant stone-forming salt in urine. These investigations were performed in synthetic urine and real urine collected from healthy black and white South African males and the following parameters were assessed: urine composition; CaOx metastable limit; particle size-volume distribution; ^{14}[C]-oxalate deposition kinetics; CaOx crystal nucleation, aggregation and growth kinetics; examination of crystalluria by scanning electron microscopy and calculation of various physicochemical risk indices (Bonn Risk Index, Tiselius Risk Index and the relative urinary supersaturation of several stone-forming salts). All plant extracts inhibited one or more of the crystallization processes.

Furthermore, crystal-cell binding, another risk factor for stone formation, was investigated in the presence of plant extracts. Madin-Darby canine kidney (MDCK)-I cells were used for this experiment. Crystals (inorganic and urinary) were bound to cells incubated in both aqueous media and real urine. Results showed that plant extracts reduced crystal binding under some but not all conditions. One of the extracts (*Folium pyrrosiae*) was administered to healthy South African black (n=9) and white (n=9) males in a double-blind placebo-controlled study. No significant effects on urine chemistry were found and there were no significant differences between the race groups post-treatment. Compounds from this herb were isolated and purified by the use of sequential liquid-liquid extractions and gel-permeation chromatography. A novel compound, *5-(3-(5,5-dihydroxy-3-oxopentyl)phenoxy)-2-hydroxy-5H-indene-6-carboxylic acid*, was identified using mass spectrometry and nuclear magnetic resonance imaging spectroscopy.

The findings in this thesis have contributed to the body of knowledge about kidney stone disease. It has been demonstrated that some herbal preparations may be potentially useful in treating and managing this disease, but further clinical testing is required prior to the implementation of such an approach.

ABBREVIATIONS

BRI	Bonn Risk Index
BU	Urine from black subjects
BuOH	Butanol
BUP1	Black urine pool 1
BUP2	Black urine pool 2
CaOx	Calcium oxalate
COD	Calcium oxalate dihydrate
COM	Calcium oxalate monohydrate
CYS	Cystone ®
DS	*Desmodium styracifolium*
EtOAc	Ethyl acetate
FP	*Folium pyrrosiae*
GAGs	Glycoaminoglycans
GC	Gas chromatography
HCl	Hydrochloric acid
HPLC	High-performance liquid chromatography
HT	*Hylocerus trigonus*
iCOM	Inorganic calcium oxalate monohydrate
l	liter(s)
M	Molarity
mM	Milli molar
mL	milliliter
MS	Mass spectrometry
MSL	Metastable limit
MSMPR	Mixed suspension mixed product removal
NaOH	Sodium hydroxide
Na_2Ox	Sodium oxalate
NMR	Nuclear magnetic resonance
OD	Optical density
OS	*Orthosiphon stamineus*
PBS	Phosphate buffer saline
PN	*Phyllanthus niruri*

ppm	Parts per million
PSD	Particle size distribution
RS	Relative supersaturation
SE	Standard error
SEM	Scanning electron microscopy
SS	Supersaturation
SU	Synthetic urine
TLC	Thin-layer chromatography
TRI	Tiselius Risk Index
uCaOx	Urinary calcium oxalate
WU	Urine from white subjects
WUP1	White urine pool 1
WUP2	White urine pool 2
XRD	X-ray powder diffraction

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General introduction

Urolithiasis, the formation of calculi in the urinary tract, has been well-established as one of the oldest and most painful diseases of mankind through different archaeological findings (*cited in:* Lopez and Hoppe 2010). Early recordings of stones have been found in Papyrus Ebers, the main origin of traditional Egyptian medicine, dating back to 1500 BC (*cited in:* Eknoyan 2004). Furthermore, in 1901, English archeologist E. Smith found a 5000 year old stone in an Egyptian mummy (*cited in*: Lopez and Hoppe 2010). During the same era, various treatments to expel or dissolve stones were identified in Mesopotamia. Saltpeter (potassium nitrate) and turpentine oil (distilled product of resin from pine trees) were known for their diuretic action, and pulverized egg shells (calcium carbonate) were ingested to complex with potential stone forming species (*cited in*: Dardioto *et al.* 1997, Shah and Whitfield 2002). Uroscopy (a historic medical practice of examining urine for symptoms of disease) was mentioned in the Hippocratic Collection five centuries BC (*cited in*: Dardioto *et al.* 1997, Sachs 2003). And three centuries BC, Ammonius introduced lithotrity to break up bladder stones so that they could be passed-out in urine (*cited in*: Shah and Whitfield 2002). Despite these revolutionary advances in combatting stone disease, surgical procedure only became popular two centuries later.

In the first century BC, ancient Greek Medicine had become popular in the Roman Empire and one of the foremost physicians of the time, Aulus Cornelius Celsus, introduced lithotomy (a surgical procedure for stone removal). However due to the absence of anesthetics, catastrophic side effects such as excruciating pain and even death ensued as a result of infection. This procedure, known as the Celsic method, was employed up until the eighteenth century (*cited in*: Shah and Whitfield 2002, Sach 2003, Lopez and Hoppe 2010).

Kidney stone disease continues to be a common modern-day medical disorder in industrialized and developing countries. However there have been several developments in understanding its pathology and treatment over the years.

The disease has also shown a greater incidence in males (12%) than in females (5%) (Blacklock 1982, Hesse *et al.* 2003, Parmar 2004). The pathogenesis of urinary stones is multi-factorial in nature (Blacklock 1982, Li *et al.* 1985, Ljunghall *et al.* 1985, Kohri *et al.* 1988, Smith 1989, Curhan *et al.* 2004b, Holmes and Assimos 1999, Goldfarb 2005, Moe and Bonny 2005, Stoller and Rubenstein 2005). However idiopathic stone formation, indicating the absence of a medical cause of the disease is also common.

Studies have shown that 70-80% of urinary stones are composed predominantly of calcium oxalate (CaOx) (Marangella *et al*, 1999, Aggarval *et al.* 2000, Coe *et al.* 2005, Khan 2006, Heilberg and Schor 2006). Consequently, the factors that govern CaOx precipitation have been a target of research over the years.

In this general introduction, four main aspects of kidney stone disease will be presented: epidemiological factors, mechanisms of stone formation and composition of stones, physiochemical urinary risk factors and methods of treatment.

1.1 Epidemiological factors

The prevalence of urolithiasis (defined as the history of stone disease) varies according to climate and geography, ethnicity, age, gender, occupation and diet (Scott 1985, Beukes *et al.* 1987, Sakhaee *et al.* 1987, Ramello *et al.* 2000, Curhan 2011). The incidence of kidney stones (defined as the first stone event) also varies in different parts of the world (Robertson *et al.* 1975) due to differences in lifestyle, diet, climate and daily water intake (Curhan *et al.* 1993, Trinchieri 2006, Hesse *et al.* 1993). In addition, other studies have related these variations in stone prevalence and incidence to co-morbidities such as diabetes, obesity and hypertension (Daudon *et al.* 2004, Leiske *et al.* 2006, Cupisti *et al.* 2007, Sakhee *et al.* 2011).

1.1.1 Climate and geography

There are some areas of Europe, South East United States (South Eastern "stone belt") and Africa where the incidence of stone formation is notably higher than the Western world average (Blacklock 1982, Ramello *et al.* 2000, López and Hoppe 2010, Romero *et al.* 2010). In these developed countries, the risk of urinary stone incidence was reported to be 13% in North America, 12% in Canada, and between 5-9% in Europe (Ramello *et al.* 2000) with the highest risk existing in Saudi Arabia, namely 20% (Robertson and Hughes 1994, Alsuwaida *et al.* 2010). It is postulated that the extremely hot climatic conditions in these areas (in addition to dietary factors) contribute to increased perspiration and dehydration leading to decreased urinary output. Hence increased concentrations of various stone forming salts and relative supersaturations thereof, all of which are known to increase the risk of stone formation (Al-Dabbagh and Fahadi 1977, Schwille and Herman 1992). Another effect of prolonged exposure of the skin to UV radiation is the conversion of 7-dehydrocholesterol to vitamin D_3. This vitamin and its hydroxylated derivative 1,25-hydroxyvitamin D_3, act on the intestine and bone to increase plasma Ca^{2+} and PO_4^{3-} levels resulting in an increase in intestinal calcium absorption thereby increasing the potential of stone formation (Robertson and Peacock 1983, Broadus *et al.* 1984, Berlin *et al.* 1986).

1.1.2 Gender and race

With respect to gender, males have a higher stone incidence than females (12% vs 5%) (Blacklock 1982, Hesse *et al.* 2003, Parmar 2004) and a higher average urinary oxalate concentration than women (Curhan 1999, Miller *et al.* 1977). These differences have been attributed to hormones in the respective sexes. Estrogen has been shown to give immunity to women by lowering the urinary supersaturation of stone-forming salts (Heller *et al.* 2002, Ferrari *et al.* 2004) whereas the increased risk in men may be due to testosterone increasing urinary oxalate levels (Lee *et al.* 1996, Yoshihara *et al.* 1999).

Worldwide, there has been a higher incidence of idiopathic stone formation reported in the white population relative to their black counterparts irrespective of other epidemiological factors (Muskat 1951, Modlin 1967, Robertson *et al.* 1980, Soucie *et al.* 1994, Whalley *et al.* 1998, Ramello *et al.* 2000, Maloney *et al.* 2005, Taylor and Curhan 2007). Similarly, a lower incidence of stones has also been found in ethnic groups like the Inuits, Australian Aborigines, American blacks and South African blacks (Modlin 1967, Bateson 1977, Whalley *et al.* 1998, Akoudada *et al.* 2010). The rarity of stones in the black population of South Africa is of great interest in the context of this thesis and will be discussed in greater detail later in the chapter.

1.1.3 Occupation

Another factor affecting susceptibility to urolithiasis is that of occupation. The prevalence of stone formation is found to be greater in people with sedentary work e.g. aviation pilots and truck drivers (Blacklock 1969, Borghi *et al.* 1993, Zheng *et al.* 2002). A possible explanation for the higher risk in truck drivers is that infrequent voiding increases the urinary concentration of the various stone forming salts. Stone disease was also found to be five times higher in outdoor workers than those who worked indoors, suggesting chronic dehydration to be a contributory factor (Pin *et al.* 1992) which is also the likely cause of a higher incidence rate in machinists who were exposed to a hot environment and massive sweating (Borghi *et al.* 1993).

1.1.4 Diet

The association of dietary factors on urine composition has been verified by many studies over the past several years (Trinchieri *et al.* 1991, Curhan 1993a, Curhan 1993b, Massey *et al.* 1993, Curhan *et al.* 1996, Rodgers and Lewandowski 2002, Siener and Hesse 2002, Massey 2003, Taylor and Curhan 2004, Goldfarb 2005, Massey *et al.* 2005, Borghi *et al.* 2006, Siener 2006, Kynast-Gales and Massey 2007, Karagűlle *et al.* 2007, Thomas *et al.* 2008, Meschi *et al.* 2011). Nutrition plays and important role in stone formation as it alters urinary composition and supersaturation. Certain foodstuffs are considered lithogenic (increases the risk of stone formation) whereas others are anti-lithogenic (decreases the risk of stone formation). Specific dietary factors implicated in stone disease include: calcium, oxalate, citrate, magnesium, animal protein, carbohydrate, sodium, phytate and fluid intake.

Calcium

Although a restriction of calcium intake was previously considered to be anti-lithogenic (Robertson *et al.* 1981, Breslau 1994, Heller *et al.* 2003), several studies have stated that the contrary is true (Bataille *et al.* 1983, Messa *et al.* 1991, Messa *et al.* 1997, Curhan 1999). While the initial hypothesis was largely based on the finding that most recurrent stone formers have hypercaliuria (Pak 1998), calcium restriction is no longer recommended as decreased calcium intake could induce a potential risk of hyperoxaluria (Bataille *et al.* 1983, Jaegaer *et al.* 1994) and bone loss which could lead to osteoporosis (Coe *et al.* 1982, Curhan *et al.* 1993a, Curhan *et al.* 1996).

Curhan *et al.* (1993) showed an inverse correlation between stone formation and intake of dietary calcium. A plausible explanation for this finding could be that by reducing the intake of dietary calcium, the binding of calcium to oxalate in the gut decreases thereby allowing more oxalate to be absorbed in the blood stream and excreted in the urine (Curhan *et al.* 1993a). For kidney stone patients, a recommended calcium intake of 800-1200 mg/day should be maintained rather than varying its consumption (Wahl and Hess 2000, Lewandowski and Rodgers 2004, Moe 2006).

Oxalate

Dietary oxalate is a crucial risk factor for calcium oxalate stone formation as it influences the concentration of urinary oxalate (Hess *et al.* 1993, Holmes *et al.* 1995, Holmes *et al.* 1998, Holmes and Kennedy 2000, Pak *et al.* 2004a, Coe *et al.* 2005). Oxalate complexes with calcium in urine to form CaOx which is the most common stone forming precipitate. Hence stone formers are advised to minimize consumption of foodstuffs containing high levels of oxalate such as: spinach, rhubarb, beetroot, peanuts, chocolates, strawberries and tea (Hesse *et al.* 1993, Massey *et al.* 1993, Holmes and Kennedy 2000). Estimates of normal dietary oxalate intake are in the range of 50-200 mg/day (Holmes *et al.* 1995, Siener and Hesse 2002, Taylor and Curhan 2007a).

Urinary oxalate is derived both exogenously and endogenously. Exogenous oxalate is absorbed from the gastrointestinal tract (Holmes *et al.* 1999) and contributes 10-40% of urinary oxalate but some variation in this figure has been reported (Finch *et al.* 1981, Williams and Smith 1983, Holmes *et al.* 1999). Factors that influence the absorption of exogenous oxalate are not clearly known but are likely to include other dietary factors (e.g. calcium), genetic factors and intestinal flora. The remaining oxalate is derived endogenously as an end-product of ascorbate metabolism and a by-product of the metabolic reaction involving glyoxalic acid (Massey *et al.* 1993, Robertson 1999).

Researchers have investigated the influence of dietary calcium on urinary oxalate and the results show that as dietary oxalate increases, urinary oxalate increases only when calcium intake is minimized (Marshal *et al.* 1972, Breslau *et al.* 1988, Hess *et al.* 1996, Heilberg 2000, Curhan 2004, Krieg 2005).

Citrate

The effect of citrate and several citrate-containing supplements as a therapeutic agent in treating and managing urolithiasis has been extensively investigated and reviewed (Pak 1987, Pak 1991, Pak 1994, Lemann *et al.* 1989, Rumenapf and Schwille 1987, Whalley *et al.* 1998, Allie-Hamdulay *et al.* 2005, Pearle *et al.* 2008).

These studies have been carried out by means of (1) in vitro crystallization experiments (Ryall *et al.* 1981, Kok *et al.* 1987, Tiselius 1993, Bek-Jensen *et al.* 1996), (2) measurement of baseline urinary citrate in healthy and CaOx stone forming subjects (Nicar *et al.* 1983, Pak *et al.* 1985, Laminski *et al.* 1990, Cupisti *et al.* 1992), and (3) clinical trials involving kidney stone patients whose urinary risk factors (Allie-Hamdulay *et al.* 2005) and stone recurrence rates were measured pre- and post-treatment with citrate-containing preparations (Pak *et al.* 1994, Barcelo *et al.* 1993, Whalley *et al.* 1996).

Citrate has been found to consistently increase urinary pH (acts as an alkalinizer) and induces an increase in the dissociation of uric acid (Schwille *et al.* 1985, Schwille *et al.* 1987, Berg *et al.* 1990, Abdulhadi *et al.* 1993, Hofbauer *et al.* 1994, Ogawa 1994, Heilberg and Schor 2006). Furthermore, its inhibitory potency lies in its ability to form soluble complexes with calcium thereby reducing urinary supersaturation of CaOx and CaP and retards nucleation, aggregation and growth of crystals thereof (Pak and Fuller 1986, Ettinger *et al.* 1997, Laube *et al.* 2002, Grohe *et al.* 2011). A recent study also showed that citrate binds specifically to the large flat surface of COM crystals which contributes to its inhibition of COM crystal growth and aggregation (Grohe *et al.* 2011).

Magnesium

Dietary magnesium is considered to be a prophylaxis in the management of urolithiasis as it binds free oxalate in the gastrointestinal tract thereby reducing its excretion in urine (Barilla *et al.* 1978, Berg *et al.* 1986, Pearle *et al.* 2008). Magnesium oxalate is more water-soluble than CaOx. In this way it can decrease the supersaturation of CaOx (Lindberg *et al.* 1990) thereby decreasing the risk of stone formation. Lower urinary magnesium excretion has been reported in stone formers compared to healthy controls (Tiselius *et al.* 1978). Some studies have shown that an increase in the oral intake of magnesium leads to an increased excretion of urinary magnesium (Fetner *et al.* 1978, Johanson *et al.* 1980a), but no effect was seen in others (Fetner *et al.* 1978, Ettinger *et al.* 1984) and gastrointestinal disorders were produced in some (Johansson *et al.* 1980b). Magnesium has also demonstrated inhibition of nucleation, aggregation and growth of CaOx and calcium phosphate crystals (Li *et al.* 1985, Achilles and Ulshöfer 1985) in *in vitro* crystallization experiments.

High fluid intake

The main determinant of urine volume is fluid intake. It is routinely recommended that stone forming patients increase their fluid intake to between 2.5-3 litres per day in order to decrease the likelihood of stone recurrence (Pak *et al.* 1984, Coe *et al.* 1992, Rodgers 1999, Borghi *et al.* 1996, Borghi *et al.* 1999, Meschi *et al.* 2004). Dehydration elevates the saturation of stone forming salts and decreases urinary pH, both of which are risk factors for stone formation.

Therefore, theoretically a high volume of fluid intake may decrease risk as it would dilute the urine thereby decreasing the concentration of stone forming constituents and lowering urinary saturation (Goldfarb 1990, Goldfarb 1994). However concern has been expressed that diluting urine would also decrease the concentration of inhibitors which may counter the effect of reduced saturation. Pak *et al.* (1980) conducted studies to clarify these conflicting theories and concluded that urinary dilution decreases crystallization of calcium salts and lowers the level of supersaturation. Hence a greater intake volume is considered to have a protective effect against stone formation (Pak *et al.* 1980). Increased urine dilution might also exert its antilithogenic effect by reducing the renal intratubular transit time thereby favoring the expulsion calcium precipitates (Pak *et al.* 1980).

Stone formers are advised to consume sufficient fluid to obtain a minimum urinary output of 2 litres per day (Goldfarb 1990). Mineral water containing solutes like calcium and magnesium have demonstrated prophylactic effects on stone formers. In a study carried out by Rodgers (1998) on male stone formers, 9 risk factors changed favourably upon consumption of mineral water rich in magnesium and calcium (Rodgers 1998).

Other beverages have shown adverse effects on stone formation e.g. coffee (Goldfarb *et al.* 2005), herbal teas (Hesse *et al.* 1993), cranberry juice (McHarg *et al.* 2003), apple juice (Vahlensieck 1986, Curhan *et al.* 1996) and lemonade (Seltzer *et al.* 1996, Kang *et al.* 2007, Penniston *et al.* 2007, Aras *et al.* 2008). However, the effect of these beverages is due to their composition i.e. the presence caffeine in tea and coffee (Goldfarb *et al.* 2005), and citrate in fruit juices such as lemonade. Beverages with a high oxalate content namely cola (Rodgers 1999) and hot chocolate (Hesse *et al.* 1993) should be avoided.

Animal protein intake

Another important dietary factor with respect to effect on stone disease is that of animal protein intake and a strong correlation exists (Robertson *et al.* 1979, Robertson *et al.* 1981, Brockis *et al.* 1982, Yoshida and Okada 1990, Curhan 1993, Hesse *et al.* 2003, Pak 2004, Siener 2006). Biochemically, a diet high in animal protein causes an increase in the urinary excretion of calcium, oxalate and uric acid and a decrease in citrate excretion. All of which are well-known risk factors for idiopathic stone formation (Curhan 1993, Trinchieri *et al.* 1991, Nguyen *et al.* 2001).

Nguyen *et al.* (2001) postulated that in approximately one third of idiopathic calcium oxalate stone formers, urinary oxalate increases after an animal protein load (Nguyen *et al.* 2001). A proposed mechanism for the increased calcium excretion is that dietary protein increases endogenous acid production. Elevated levels of acid stimulate an increase in calcium reabsorption of bone hence increased urinary calcium excretion (Brockis *et al.* 1982). Another hypothesis for the increased urinary calcium is that sulphur-containing amino acids (predominant in animal protein) complex with calcium, preventing its reabsorption by renal tubules thereby increasing its urinary excretion (Schulette *et al.* 1980). A high animal protein load increases urinary uric acid due to its high content of purine, a substrate for urate synthesis, (Coe *et al.* 1976, Krieg 2005) resulting in transient acidosis thereby increasing tubular reabsorption of citrate and lowering urinary citrate (Fellström *et al.* 1984, Siener and Hesse 2002).

Carbohydrate

Some researchers claim that the higher incidence of stones in wealthier countries is due to increased refined sugar intake (Robertson *et al.* 1978a, Robertson *et al.* 1978b, Thorn *et al.* 1981, Robertson *et al.* 1987). A correlation has been observed between an increased carbohydrate intake and an increased excretion of urinary calcium in both healthy subjects and CaOx stone formers (Wood and Allen 1983, Iguchi *et al.* 1993, Coe and Parks 1994b, Leman *et al.* 1989, Taylor and Curhan 2004) although this increase was more prominent in the latter group. Furthermore, refined sugars also stimulate endogenous synthesis of oxalate which is eventually excreted in urine (Lekcharoensuk *et al.* 2001). High urinary concentrations of calcium and oxalate pose a serious risk for stone formation as these are the main urinary CaOx risk factors.

Therefore a low carbohydrate diet is recommended to stone-forming patients (Lekcharoensuk *et al.* 2001).

Sodium

A high dietary sodium intake is a risk factor for stone formation due to its calciuric action independent of calcium intake (Kok *et al.* 1990, Sakhaee *et al.* 1993, Pak *et al.* 2004). It has been reported that a 100 mmol increase in daily sodium intake is associated with an approximate increase of 1 mmol in urinary calcium excretion (Nordin *et al.* 1993, Lemann *et al.* 1999). This effect is probably due to the inhibition of sodium and calcium reabsorption in the proximal tubule and along the loop of Henle (Curhan 2004). Other studies have found that increasing dietary sodium caused a decrease in urinary citrate (Kok *et al.* 1990, Sakhaee *et al.* 1993). Hypercalciuria and hypocitraturia are important lithogenic consequences of an excessive intake of dietary salt, thereby increasing the risk of CaOx stone formation.

Phytate

Phytic acid (inositol 1,2,3,4,5,6-hexakisphosphate) is a natural sugar present in most seeds and cereals. The compound contains six phosphate groups, each with two hydroxyl moieties. In its ionic state the molecule is able to chelate with divalent metal ions, e.g. Zn^{2+}, Mg^{2+}, Fe^{2+} and most importantly Ca^{2+}, to form insoluble complexes (Ryall 2011). Several studies have reported that phytate is one of the most powerful inhibitors of CaP and CaOx crystallization (Grases and March 1989, Graf and Eaton 1990, Grases *et al.* 1994, Grases *et al.* 2000a, Grases *et al.* 2000b). The concentration of phytate in normal human urine has been reported to be 2.5 mg/L which is significantly higher than that of stone formers (1.5 mg/L) (March *et al.* 1998, Grases *et al.* 1999). There is an important positive correlation between dietary intake and urinary excretion of phytate (Taylor and Curhan 2004). A clinical study conducted by Conte *et al.* (1989) on CaOx stone formers showed that even low consumption of phytate significantly reduced the risk of developing stones (Conte *et al.* 1989c). The potency of phytate in reducing risk of stone formation rests in its calcium binding affinity (March *et al.* 1998, Grases *et al.* 2000a).

1.2 Physicochemical mechanisms of stone formation and composition of kidney stones

Stones in the urinary tract are predominantly crystalline with the rare exception of matrix stones and there are several important physiochemical mechanisms involved in stone formation which include CaOx supersaturation and CaOx crystal nucleation, growth and aggregation (Finlayson 1978, Rose 1982, Coe *et al.* 1992, Hess and Kok 1996, Coe and Parks 1997, Kavanagh 2006a, Kavanagh 2006b).

1.2.1 Supersaturation

Supersaturation, the fundamental driving force for the change of phase from solution to solid (i.e. when crystallization occurs), is the first and most essential step in the process of stone formation. It is an expression of the excess of free energy (**ΔG**) between two phases, i.e. the difference in chemical potential of the solution (μ_{soln}) and the chemical potential of the crystalline phase at equilibrium (μ_{cryst}). A positive change in the chemical potential ($\mu_{soln} - \mu_{cryst}$) favours crystallization whereas a negative chemical potential favours dissolution. Chemical potential can be expressed in terms of the gas constant (**R**), the absolute temperature (**T**), the activity of the unionized salt in the supersaturated solution (**A**) and the activity of the solution at equilibrium ($\mathbf{A_{eq}}$) (Finlayson 1978, Hess and Kok 1996, Kavanagh 2006a) as follows:

$$\Delta G = \mu_{soln} - \mu_{cryst} = RT \ln (A/A_{eq})$$

The supersaturation ratio ($\mathbf{A/A_{eq}}$) is referred to as the relative supersaturation (**RS**). When RS < 1, the solution is undersaturated and stone forming species remain in solution. A solution for which RS = 1, is said to be saturated and an equilibrium exists between the salts that have crystallized out and those in solution. When RS > 1, the solution is said to be supersaturated in which case crystals can grow (Finlayson 1978, Hess and Kok 1996, Coe *et al.* 2005). It has been reported that urine from stone formers tend to be more supersaturated than those of healthy individuals (Robertson *et al.* 1968, Marangella *et al.* 1985, Coe and Favus 1986). However other researchers have reported the contrary, namely that people who have never passed a stone in their life sometimes pass highly saturated urine (Ryall 1993, Hess and Kok 1996, Kavanagh

2006a). Therefore supersaturation of urine is not a pathological problem as long as crystalline particles pass freely through the urinary tract. A pathological scenario arises when crystals are retained and begin to grow (Finlayson and Reid 1978a, Kok and Khan 1994).

1.2.2 Nucleation

The first kinetic step in stone formation is nucleation i.e. the phase transformation from liquid into solid in a supersaturated solution. When this process occurs, stone forming salts in solution form loose clusters which can increase in size due to the addition of new clusters. These clusters form the substrate upon which growth and aggregation can occur (Finlayson 1978, Brown and Purich 1992, Kok and Khan 1994).

Urine lying between the solubility product (S<1) and the formation product (S>1) is referred to being in a zone of metastability or metastable (Nordin *et al.* 1993). When urine surpasses the upper limit of metastability, the solution becomes unstable and crystals form prolifically (Robertson *et al.* 1981). This spontaneous formation of crystals is known as homogenous nucleation which actually very rarely occurs in urine due to the presence of other foreign nucleating material (e.g. cell debris, urinary macromolecules and crystalline material of other stone forming salts). Heterogeneous nucleation is more likely as most stones are a mixture of more than one crystal type and different proteins (Hess and Kok 1996). There are two phenomena closely associated with heterogeneous nucleation i.e. secondary nucleation and epitaxy. The former describes the nucleation of crystals on pre-existing surfaces of the same material. Epitaxy refers to the compatibility of one crystalline material precipitating upon the surface of another whose lattice dimensions match (Hess and Kok 1996).

Urinary supersaturation can be calculated using the computer program, EQUIL. This program takes into account the chemical analysis of 23 urinary components and the calculation of 103 complexes (Werness *et al.* 1985, Brown *et al.* 1993). More recently, another chemical speciation program Joint Expert Speciation System (JESS), has been used to determine speciation and saturation index (SI) values (Rodgers *et al.* 2007). JESS has a large database comprising over 70 000 chemical reactions involving 200 000 thermodynamic constants related to enthalpy,

entropy, Gibbs free energy, solubility products and redox potential and can be used to model chemical speciation under any specific conditions (May and Murray 1991a, May and Murray 1991b).

1.2.3 Crystal growth

Crystal growth is the addition of new crystal components onto an existing crystal nucleus i.e. the incorporation of solute into the crystal lattice (Hess and Kok 1996). The kinetics of crystal growth can be described by the following differential equation (Gill *et al.* 1984):

$$- (dS/dt) = (dP/dt) = kAS^n$$

where **S** = supersaturation, **t** = time, **P** = precipitation, **k** = reaction rate constant, **A** = surface area for crystallization, **n** = order of the reaction. For CaOx, n = 2. Therefore after integration, the above equation becomes:

$$– (1/s) = kAt – (1/S_o)$$

where S_o = initial supersaturation. Therefore by keeping the surface area constant in metastable supersaturated solutions, the rate constant (k) can be derived. This can be utilized in the Langmuir adsorption isotherm to compare inhibitory activities of added substances (Gill *et al.* 1984). In whole urine, calcium oxalate crystallization can be directly measured by adding specific quantities of oxalate seed crystals and measuring changes of ^{14}C in the supernatant fraction (Gill *et al.* 1984).

While nucleation is undoubtedly a crucial first step in crystallization, it is crystal growth that induces a pathological situation. The transit time of urine through the kidney in healthy individuals is not sufficiently long enough to allow crystals to grow to a size range which will allow them to be trapped in the renal tubules (Finlayson *et al.* 1984, Hess and Kok 1996). However if crystals grow too big and become trapped in the renal tubule, they disturb the flow of

urine and provide a substrate to which newly formed urinary crystals attach and further increase in particle size occurs (Brown and Purich 1992, Hess and Kok 1996).

1.2.4 Aggregation

Crystal aggregation is the process whereby crystalline particles in solution stick together to form a larger particle. It is a natural process which is energetically favoured and can occur in all states of saturation (Kok and Khan 1994, Hess and Kok 1996). Aggregation of crystals is achieved through several electrostatic interactions, namely van der Waals forces, viscous binding and solid bridges. Attractive van der Waals forces favour particle aggregation and increases strongly as the distance between particles decreases. Viscous binding refers to the sticky properties of foreign molecules, such as macromolecules, which stick to crystal surfaces and encourage aggregation. Solid bridges refer to crystalline material connecting two particles which stabilizes an aggregate (Hess and Kok 1996).

A force which has opposing effects i.e. can promote both aggregation and disaggregation, is the shear force arising from stirring crystalline solutions or by solvent currents in tubular fluids. This dual force can favour aggregation by increasing the collisions between particles but it can also have a counter effect by disrupting particles (Hess and Kok 1996).

The zeta potential is the electrical potential at the interface between the solid particle and the surrounding solution. In the field of urolithiasis, zeta potential has been used to study the mechanism of aggregation where an increase in the negative charge of the zeta potential is interpreted as an increase in inhibition of aggregation (Finlayson *et al.* 1984, Scurr *et al.* 1986, Hess *et al.* 1989, Cao *et al.* 1996).

Several studies have reported that aggregation is the most important step in stone formation. Evidence in support of this is that the short transit time of the urinary system is insufficient for CaOx crystals to grow into a significant size to be problematic whereas aggregation can occur within seconds. Furthermore, microscopic and ultrastructural studies of kidney stones show that stones are highly aggregated structures (Meyer *et al.* 1971, Hess and Kok 1996).

1.2.5 Urinary inhibitors

As mentioned previously that under normal conditions urine is supersaturated and sometimes even CaOx crystallization occurs. However, stones do not form in all cases due to the presence of inhibitors that retard the processes of stone genesis (Boyce *et al.* 1968, Fleisch 1978, Rose *et al.* 1982, Hess and Kok 1996b, Worcester 1996, Ryall 1997, Khan and Hackett 1993, Khan and Kok 2004, Ryall 2004). Inhibitors may function by preventing nucleation, or if crystals do form, they may hinder enlargement by growth and aggregation. Furthermore, they can affect crystal-cell binding interactions or induce dissolution of endocytosed precipitates (Ryall 2011).

Inhibitors are classified into 2 categories, namely macro- and small chemical species. Macromolecular inhibitors generally refer to glycosaminoglycans (GAGS) and proteins, while the smaller inhibitors include citrate, magnesium, pyrophosphate and phytate.

Glycosyaminoglycans are unbranched polysaccharides made up of repeating dissacharide units. Each unit consists of an N-acetyl-hexosamine (amino sugar) and a hexose or hexuronic acid which are linked together by a glycoside bond. The molecular weight of GAGS vary from 18-48 kDa (Nishio *et al.* 1985, Roberts and Resnick 1986). Examples of urinary GAGS are chondroitin sulfate (CS), heparan sulphate (HS), keratan sulphate (KS), dermatan sulphate (DS) and non-sulphated hyaluronic acid (HA) (Khan and Kok 2004). In various studies, these macromolecules have been reported to inhibit nucleation, aggregation and growth of CaOx crystals (Koide *et al.* 1981, Sallis *et al.* 1981, Ryall and Marshall 1984, Breslau *et al.* 1994, Khan and Kok 2004) however unequivocal proof of their mode of action has yet to be established (Ryall 2011).

Osteopontin is a phosphoprotein with a molecular weight of between 44 to 75 kDa (Malyankar *et al.* 1997). OPN has been reported to be an inhibitor of nucleation, growth and aggregation of CaOx crystals (Asplin *et al.* 1998, Wang *et al.* 2008, Mo *et al.* 2007). In addition to its inhibitory activity, OPN demonstrated the ability to promote crystallization of COD (calcium oxalate dihydrate) in preference to COM (calcium oxalate monohydrate). COD is known to have a lower affinity for adhesion to renal epithelial cells (Wesson *et al.* 1998) hence minimizing the risk of stone formation.

Tamm-Horsfall protein has been classified as the most abundant protein in human urine with a molecular weight of 80-100 kDa (Ryall 1997). THP is known to exist in monomeric and polymeric states and its inhibitory effect depends on its state of aggregation as the monomeric state considered to be a more potent inhibitor (Hess *et al.* 1989). There have been some conflicting theories on function of THP as it has been reported to inhibit and promote CaOx crystallization depending on the experimental conditions (Hess 1994, Grover *et al.* 1998), however it has been shown to strongly inhibit COM aggregation (Viswanathan *et al.* 2011).

Albumin is found in great quantities in urine, urinary stones and crystal matrix (Atmani *et al.* 1998, Fraij 1989). In some experiments it has been reported to promote CaOx nucleation but inhibits CaOx crystal aggregation (Grover *et al.* 1998, Worcester 1996, Khan and Kok 2004).

Urinary prothrombin fragment-1 is found more abundantly in the kidneys of stone formers (Doyle *et al.* 1991, Doyle *et al.* 1995). The protein has a molecular weight of 31 kDa and contains approximately 154 amino acids of which 23% are glutamic and aspartic acids. In addition, 10 of the carbonic acids are γ-carboxylated. The presence of γ-carboxyglutamate (Gla) residues imparts inhibitory activity to UPTF1 due to its ability to bind calcium (Doyle *et al.* 1991, Grover and Ryall 2002, Webber *et al.* 2002, Cook *et al.* 2008). Despite the intense interest in the inhibitory functions of this molecule, conclusive evidence of its impact on stone formation has not yet been elucidated (Ryall 2011).

Bikunin is a complex plasma protease inhibitor that belongs to a family of inter-α-inhibitor proteins (Salier *et al.* 1996). It has been reported to be an inhibitor of CaOx crystallization and inhibits the binding of CaOx to renal epithelial cells (Ebusino *et al.* 1999a, Yang *et al.* 2005). But as with all urinary inhibitors, there is some uncertainty with respect to its mode of action (Ryall 2011).

1.2.6 Composition of kidney stones

The chemical compositions of stones vary depending on the ionic imbalance in the urine at the time of crystal nucleation and growth (Coe *et al.* 2005, Moe 2006). There are four main stone types: calcium (~80%), uric acid (9%), magnesium ammonium phosphate (10%) and cystine (1%) (Finlayson 1978).

CaOx stones

COM (whewellite) and COD (weddelite) stones occur more frequently than calcium oxalate trihydrate (COT) stones (Herring 1962, Streit *et al.* 1998, Grases *et al.* 1998, Bithelis *et al.* 2004, El-Shall *et al.* 2004). The vast majority of CaOx stone formers suffer from no systemic disease (Coe *et al.* 2010). Hypercalciuria (greater than 10 mM per 24 hour urine) is the most common cause of calcium stone production but stone formation may also occur as a result of primary hyperparathyroidism (elevated parathyroid hormone levels) or disorders of calcium metabolism (Coe *et al.* 2005). Stones can be pure or mixed with calcium phosphate, uric acid or ammonium urate (Trinchieri *et al.* 2005).

Calcium phosphate stones

In the majority of kidney stones, CaOx is the main constituent and CaP is present in amounts ranging from 1%-90% (Mandel and Mandel 1989). When stones are made up of more than 50% calcium phosphate, they are referred to as CaP stones (Coe *et al.* 2005). CaP stones exist in various forms such as apatite (calcium hydroxyl phosphate) and brushite (calcium hydrogen phosphate) (Parks *et al.* 2004, Worcester and Coe 2008). It usually occurs in patients with hormonal disorders such as hyperparathyroidism or metabolic disorders such as renal tubular acidosis (a condition whereby the kidneys are unable to excrete acid) (Hess and Jaeger 1993). High urinary pH and low urinary citrate are the driving forces for the formation of CaP stones (Pak *et al.* 1978, Parks *et al.* 2004).

Uric acid stones

Uric acid is the end product of purine metabolism and approximately 3% of stone-formers form this type of stone (Nordin and Hodgkinson 1967). Low urinary pH (below 5.5) is dominant factor leading to the formation of uric acid stones. Other contributing factors of uric acid stone formation are urate/uric acid renal handling, hyperuricosuria (high levels of urinary uric acid) and excess dietary protein (William-Larson 1990, Shekarriz and Stoller 2002, (Coe *et al.* 2005)).

Magnesium ammonium phosphate stones

Magnesium ammonium phosphate (struvite) stones are formed as a result of urinary tract infections which are caused by micro-organisms (e.g. *Proteus*, *Providencia*, *Klebsiella*, *Pseudomonas* and *Enterococci*) (Bruyeni *et al.* 2008, Gómez-Nùňez *et al.* 2009). These bacteria produce the enzyme urease which degrades urea to ammonia and carbon dioxide thereby raising urinary pH and favouring the formation of struvite. The typical morphology of struvite stones are 'staghorn' calculi (Ramello *et al.* 2000).

Cystine stones

Cystine stones represent only a small percentage (1-2 %) of urinary calculi (Ramello *et al.* 2000, Coe *et al.* 2005). These stones form as a consequence of cystinuria i.e. an inherited genetic defect that causes an abnormal transport of the amino acids cystine in the kidney and gastrointestinal system (Coe *et al.* 2005). This type of stone is commonly found in children and young adults. They grow rapidly and have high recurrence rates (Font-Llitjós *et al.* 2005).

1.2.7 Pathways of stone formation

As mentioned previously, supersaturation is the driving force of uric acid and cystine stones, and infection stones form as a result of bacterial metabolism (Moe 2006). However there is no simple theory to explain the mechanisms of the crystallization processes that lead to the formation of calcium containing calculi, the most common type (Coe *et al.* 2010, Knoll 2010). A significant component of this process is particle retention which allows the calculus to develop (Finlayson and Reid 1978a, Coe *et al.* 2005, Coe *et al.* 2010, Evan *et al.* 2010, Knoll 2010, Tiselius 2011). By use of human tissue biopsies and intra-operative imaging, 3 pathways that lead to stone formation have been identified namely (1) overgrowth on interstitial apatite, (2) crystal deposits in renal tubules and (3) free solution crystallization (Coe *et al.* 2010).

Overgrowth on interstitial apatite

More than seventy years ago, Randall discovered calcifications within the renal papilla in 20% of 1000 random cadaveric renal units (Randall 1940). These calcium salt deposits (plaques), which manifest as irregular whitish legions on the tip of the renal papillae, were interstitial (intercellular), composed of apatite (mineral phase found in bone) and were not observed in the tubular lumen. He proposed that the plaques were precursors of urinary stones (Randall 1940). Randall's hypothesis had not progressed for decades until Evan *et al.* (2003, 2005, 2006) were able to show that such CaP plaques are present in all idiopathic stone formers (Evan *et al.* 2003, Kim *et al.* 2005, Matlaga *et al.* 2007); however plaque formation is not limited to stone formers only (Evan *et al.* 2003).

Detailed examination of human tissue has shown that plaque forms at the basement membranes of the thin limbs of the loop of Henle and serves as the attachment site for stones (Matlaga *et al.* 2007, Gambaro *et al.* 2009, Coe *et al.* 2010, Evan *et al.* 2010). Matlaga *et al.* (2005) cited a positive correlation of the frequency of stone recurrence and the surface area of papillae covered by plaques (Kim *et al.* 2005) suggesting that plaque favours CaOx overgrowth (Coe *et al.* 2005). Kuo *et al.* (2003) found independent correlations between plaque surface area and *urinary volume* (inverse), *urinary calcium* (direct) and *pH* (inverse) (Kuo *et al.* 2003). No other urinary

parameter (citrate, oxalate and phosphate) was found to have a significant effect of plaque (Kuo *et al.* 2003).

Although the site of stone formation has been identified, the cascade of events of crystallization following thereafter still remains unclear (Coe *et al.* 2010, Evan *et al.* 2010, Knoll 2010, Tiselius 2011). It is suggested that urinary supersaturation is the driving force which allows crystallization to extend beyond the matrix (Coe *et al.* 2005, Coe *et al.* 2010). This pathway is primarily associated with idiopathic CaOx stone formers but has also been found in brushite stone formers and patients with primary hyperparathyroidism, ileostomy and bowel resection (Coe *et al.* 2010).

Crystal deposits in renal tubules (Intratubular crystallization)

Apart from idiopathic CaOx stone formers, all other type of stone formers are known to form crystals in their renal tubules which may lead to stone formation (Coe *et al.* 2010). These crystals project into the urinary space leading to dilation of the ducts, interstitial fibrosis and crystal mediated cellular injury (Matlaga *et al.* 2005, Coe *et al.* 2010).

Although caliceal (final) urine and urine in the distal region of the collecting duct is highly supersaturation with respect to CaOx, there is no driving force for CaOx precipitation at nephron levels above the above the collecting duct (Kok and Khan 1994, Kok and Khan 1995, Hojgaard *et al.* 1996, Tiselius and Hojgaard 1999,). However, a high level of CaP supersaturation is found in the ascending loop of Henle and distal region of the distal tubule (Deganello *et al.* 1990, Hojgaard *et al.* 1996, Tiselius and Hojgaard 1999, Evan *et al.* 2003) and microscopic examination have confirmed the presence of crystals in these nephron segments (Resnick *et al.* 1978, Asplin *et al.* 1991, Asplin *et al.* 1996, Evan *et al.* 2007). Although these particles have not been identified by means of chemical composition analysis, study of its morphology strongly suggests CaP nuclei (Smith *et al.* 1983, Hering *et al.* 1988). There are two possible subsequent pathways that have been identified for precipitated CaP crystals nephron i.e. the interstitial route where precipitates are internalized by tubular cells or the intratubular route where crystal deposits are transported down the urinary stream towards the collecting duct and caliceal space (Tiselius 2011).

Internalization or endocytosis (interstitial route) is proposed to be a defense mechanism whereby cells in the nephron eliminate intratubular precipitates to prevent obstruction of tubules (Vervaet *et al.* 2010). Dissolution of crystals may be brought about by lysosomes and macrophages (Smith *et al.* 1983). Alternatively, CaP crystals move down the nephron at a slower rate (Khan and Hackett 1991, Khan 1995, Yuen *et al.* 2010) which allows time for aggregation to occur. If these aggregates are too large to be handled by the cells it remains intratubular. It has been suggested that these crystals are translocated to the basement membrane/interstitial tissue through cells or between cells (Evan *et al.* 2007, Coe *et al.* 2010, Tiselius 2011).

Free solution crystallization

The most common stones formed via this pathway are composed of cystine. Cystine is poorly soluble and highly supersaturates urine in the ducts of Bellini which leads to stone formation. These stones are always found free and unattached in the renal pelvis (Coe *et al.* 2010). Even though it is an uncontroversial pathway, it's still of great importance as it illustrates how the most simplified mechanisms of crystallization can give rise to complex stone formation.

1.3 Urinary risk factors

As mentioned, various epidemiological factors affect the risk of stone formation. These parameters influence one or more of six urinary risk factors (Robertson and Peacock 1983) and are considered to be the main determinants of CaOx crystallization (Robertson *et al.* 1978, Finlayson *et al.* 1984, Hess *et al*, 1996). Robertson and Peacock (1983) identified these risk factors to be hypercalciuria, hyperoxaluria, hyperuricosuria, elevated pH, decreased volume and decreased excretion of urinary inhibitors (i.e. magnesium, citrate, phosphate and pyrophosphate) (Robertson and Peacock 1983).

1.3.1 Urinary calcium

It is well-known that abnormally high calcium levels (hypercalciuria) is a risk factor for recurrent stone formation as it contributes to the supersaturation of both CaOx and CaP stones (Goldfarb 1994, Borghi *et al.* 1996). Many studies have found a notably higher incidence of idiopathic hypercalciuria in stone formers than in healthy subjects (Coe *et al.* 1982, Pak 1998, Heilberg 2000, Curhan *et al.* 2001). Researchers have reported the normal urinary calcium excretion upper limit to be 7.5 mmol per 24 hours in men and 6.25 mmol in women. A 24 hour urinary calcium excretion reaching 10 mmol poses the threat of stone formation (Robertson *et al.* 1978, Monk 1996, Bihl and Meyers 2001).

Hypercalciuria presents itself in stone formers in one of three ways, namely: idiopathic (absorptive), resorptive and renal.

Idiopathic (absorptive) hypercalciuria is the most prevalent type (30 – 50% of patients) and is caused by an overproduction of 1,25-dihydroxy-vitamin D_3. This molecule is responsible for the transportation of calcium ions through the intestinal cells. Therefore patients with this condition absorb far more calcium from foodstuffs than healthy subjects which leads to elevated serum calcium levels resulting in high urinary calcium excretion (Jaegar 1998, Parmar 2004, Bushinsky 2002, Coe *et al.* 2005).

Resorptive hypercalciuria is a consequence of increased bone resorption which occurs as a result of primary hyperparathyroidism i.e. an excessive secretion of parathyroid hormone, PTH. The principal functions of PTH are to mobilize calcium from bone, conserve calcium in the kidney and indirectly increase gastrointestinal calcium absorption. This abnormality is only reported in 5% of patients with recurrent stones (Mollerup *et al.* 2002, Parmar 2004).

Renal hypercalciuria is an outcome of impaired renal tubular absorption of calcium (Parmar 2004; Coe *et al.* 2005). This condition exists in about 2% of patients with recurrent stone formation.

Various studies have also established that a high intake of sodium can also lead to elevated urinary calcium excretion as calcium and sodium have similar renal handling mechanisms (Hesse *et al.* 1993, Sakhaee *et al.* 1993, Martini *et al.* 1998). Another contributing factor to hypercalciuria is a high intake of dietary calcium (Broadus *et al.* 1978, Massey and Kynast-Gales 1998, Messa *et al.* 1997).

1.3.2 Urinary oxalate

Oxalate is a dicarboxylate anion and when complexed with calcium in urine forms an insoluble salt. At neutral pH, CaOx has a solubility of 0.67 mg/L in water, however urine is often supersaturated with respect to this salt, therefore crystallization can occur. Excessive oxalate in the urine (hyperoxaluria) is reported to be the cause of 30% of calcium stones (Bihl and Meyers 2001). There have been conflicting reports on differences in urinary oxalate between healthy subjects and stone formers. Several studies have reported a higher concentration of urinary oxalate in the latter group (Robertson *et al.* 1978, Schwille *et al.* 1989, Wilson *et al.* 1989) while others have reported no differences between the two groups (Tiselius 1997, Holmes *et al.* 1998).

The amount of oxalate excreted in urine is mainly dependent on one of four factors: (1) dietary intake, (2) intestinal absorption and renal tubular secretion, (3) the rate of endogenous synthesis and (4) a deficiency in oxalate-degrading bacteria, *Oxalobacter formigenes* (Robertson 1999, Ogawa *et al.* 2000). (1) *Increased dietary intake* of oxalate rich foodstuffs provides more

oxalate for absorption (Robertson 1999). (2) *Intestinal absorption* is an important determinant in oxalate excretion and one of the factors that promote it is malabsorption which is the inability of the intestinal tract to absorb fatty acids and other nutrients properly. It may cause calcium to bind unabsorbed fat instead of oxalate resulting in excess oxalate being absorbed in the digestive tract and eliminated through the kidneys (Earnest *et al.* 1974). (3) *The rate of endogenous synthesis* is not considered to be a likely cause of elevated levels of urinary oxalate in idiopathic stone formers (Massey *et al.* 1993). (4) *A deficiency in Oxalobacter formigenes*, a colonic anaerobe which constitutes the normal component of human microflora, can cause an increase in the risk of hyperoxaluria and stone formation. By utilizing oxalate in the digestive tract, these oxalate-degrading bacteria maintain a homeostatic oxalate balance and prevent excessive enteric oxalate absorption (Allison *et al.* 1986, Sidhu *et al.* 1999).

Hyperoxaluria is considered to be the most critical determinant of renal stone formation compared to other risk factors like hypercalciuria, hyperuricosuria (a high urinary uric acid concentration) and hypocitraturia (low urinary citrate excretion) (Robertson 1999).

1.3.3 Urinary magnesium

Magnesium has been well-established as an inhibitor of CaOx crystallization as it readily forms highly soluble complexes with oxalate thereby reducing free oxalate in the urine (Rushton and Spector 1982, Trinchieri *et al.* 1992). This is highly favourable as oxalate plays a greater role in CaOx stone formation than calcium. Apart from ability to chelate to oxalate, magnesium has been found to decrease the nucleation and growth rates of CaOx crystals in vitro as well as lower urinary supersaturation of CaOx (Ryall *et al.* 1981, Li *et al.* 1985, Lindberg *et al.* 1990).

There have been conflicting reports on the relative levels of urinary magnesium concentration in healthy subjects and stone formers. Some studies have reported no major differences between the groups (Robertson *et al.* 1968, Welshman *et al.* 1975) while others have found urinary magnesium levels to be lower in stone formers than healthy subjects (Trinchieri *et al.* 1991, Deshmukh and Khan 2006).

1.3.4 Urinary citrate

The association of hypocitraturia and stone disease has been well-documented (Pak *et al.* 1985, Laminski *et al.* 1990, Cupisti *et al.* 1992). Citrate complexes with ionic calcium thereby lowering its concentration and reducing the potential for the formation calcium precipitates. Calcium citrate complexes are also highly water soluble. Apart from this function, *in vitro* studies have demonstrated citrate to be an effective inhibitor of CaOx nucleation, aggregation and of crystal growth (Tiselius *et al.* 1990, Bek-Jansen and Tiselius 1991, Schwille *et al.* 1999). It is also known to bind to CaOx crystals surface thus influencing aggregation and growth (Ryall 1997). In view of these findings, low urinary citrate excretion is considered to be an important risk factor for renal stone formation and alkali therapy is often recommended to patients (Parks and Coe 1986, Conte *et al.* 1989a).

1.3.5 Urinary uric acid

Hyperuricosuria is another significant risk factor for renal stone formation (Robertson *et al.* 1978, Coe 1983, Griffith *et al.* 1986). Uric acid stones may be dissolved by increasing the pH to approximately 6.5 (Hesse *et al.* 1997). It has been reported that one third of patients with CaOx stones have high uric acid excretion. There are two possible causes of this namely an endogenous overproduction of uric acid (70% of cases) or a high intake of dietary protein (30% of cases) (Coe 1978). Uric acid is an end-product of purine (animal protein) metabolism. Therefore recurrent stone formers are advised to restrict their intake of foods rich in protein, such as: meat and fish (Coe 1978, Scheinman 2000). Allopurinol therapy has been reported to be very effective in decreasing urinary uric acid and recurrent stone formation by blocking uric acid production (Ettinger 1991) and purine reabsorption (Pak *et al.* 1978).

1.3.6 Urinary phosphate

The concentration of urinary phosphate is directly dependent on the dietary intake of phosphate rich foods and is therefore subject to huge variations. It has a great influence on establishing the degree of calcium phosphate crystallization (Kok *et al.* 1988). The normal urinary excretion of

phosphate is 35 mmol per twenty four hours (Hesse *et al.* 1997). Elevated levels of urinary phosphate can pose a risk for CaP crystallization especially if hypercalciuria is present. Urinary phosphate can also serve as an inhibitor of CaOx crystallization as it can complex with ionic calcium thereby reducing the availability of calcium to bind oxalate (Schwille 1989, Baumann *et al.* 2000).

1.3.7 Urinary pyrophosphate

Pyrophosphate has been reported to inhibit nucleation (Grases *et al.* 1989a, Ryall 1997), growth (Grases *et al.* 1989b, Conte *et al.* 1989b) and aggregation of CaOx crystals (Robertson *et al.* 1973, Robertson *et al.* 1974). The inhibitory activity of pyrophosphate is due to its capacity to selectively and irreversibly bind to the surfaces of COM crystals and not to COD crystals (Wikström *et al.* 1983, Wesson *et al.* 1998). This attribute is of great pathological significance as COD crystals are less likely to adhere to renal epithelial cells and crystal-cell binding is a critical step in stone formation (Wesson *et al.* 1998).

1.3.8 Urinary pH

Urinary pH is crucial in determining the ion-product activity of CaP and CaOx and hence their risk of precipitation (Pak 1994). At high urinary pH (> 7.0), more phosphate and citrate ions are dissociated which minimizes the risk of CaOx crystallization, as calcium and citrate ions are able to complex (Tiselius *et al.* 1978, Pak 1994, Højgaard *et al.* 1999). However, this inadvertently raises the risk of calcium phosphate crystallization. This was shown in a study where calcium phosphate crystals precipitated at a pH of 6.45 but no CaOx crystals formed (Højgaard *et al.* 1999) however CaOx crystallization is known to occur at pH < 6.25 (Ahlstrand *et al.* 1984). Low urinary pH (< 6.0) has been associated with excessive uric acid crystallization and subsequent stone formation (Hess 2006). Magnesium ammonium phosphate stones are reported to form at urinary pH > 7.0 due to the presence of urease producing bacteria (Hesse *et al.* 2002). Ammonium urate stones, which are also caused by infection, occur at pH 6.5 and 7.0 (Hesse *et al.* 2002). It is recommended that urinary pH be maintained between 6 and 7 in order to

minimize the risk of CaOx, CaP and uric acid stone formation (Ahlstrand *et al.* 1984, Tiselius 2003).

1.3.9 Urinary volume

Urinary volume is another crucial factor affecting renal stone formation. A high fluid intake is the first line of treatment recommended for stone formers as it reduces the relative supersaturation of crystal components in urine. A high urinary volume also aids in flushing out the renal system of any crystals that may have formed thereby reducing the risk of crystal-cell adhesion (Curhan *et al.* 1993, Borghi *et al.* 1996, Pearle *et al.* 2008). Although diluting the urine is advantageous, excessively high intake of fluids is not advised as it could decrease the concentration of inhibitors and counter its positive effects (Pak *et al.* 1980).

In studies by Borghi *et al.* (1996, 1999) the clinical significance of a high fluid intake was demonstrated. When the fluid intake was increased to achieve an output of 2 liters, both urinary calcium and urinary oxalate concentrations decreased significantly, and a reduction in stone recurrence from 27% to 12% was noted. They also reported a decrease in CaOx supersaturation but no substantial change in CaOx MSL was found. A urinary volume of 2 litres also did not affect the inhibitory properties of macromolecules on CaOx crystallization (Borghi *et al.* 1996, 1999). The overall conclusion is that a low urine volume is a risk factor of stone formation as it increases the saturation of stone forming salts (Sakhaee *et al.* 1980).

1.4 Conservative treatment and management of idiopathic CaOx urolithiasis

1.4.1 General

It is common knowledge that stone patients are very likely to have recurrent episodes, ranging from 40% within 3 years, 74% within 10 years to 98% within 25 years (Sutherland *et al.* 1985, Milliner 1995, Robertson 1999, Atmani *et al.* 2000, Johri *et al.* 2010). Despite years of medical research, there is no ideal drug to cure renal stone disease. Patients tend to be treated with the same medicines for long periods of time and efficacy of the medicines and side-effects may sometimes become problematic (Koide *et al.* 1995, Atmani *et al.* 2000, Glover *et al.* 2011, Navaneethan *et al.* 2011).

The single most common factor that initiates stone formation is insufficient fluid intake (Hall 1995, Moe 2006), therefore increased hydration is the first line of therapeutic defence. Thiazide diuretics (hydrochlorothiazide or chlorthalidone) have been reported to be effective in managing kidney stones (Yendt and Cohanim 1978, Ramsay 1999, Pearle *et al.* 2008, Eisner 2009, Goldfarb 2009, Vigen 2010). Thiazides act by blocking the reabsorption of sodium in the distal tubules of the kidney hence the laminar fluid is more concentrated (Coe 1977).

Since diet significantly affects urine composition, certain foods and dietary supplements are used as anti-lithogenic agents (Lewandowski *et al.* 2001, Lewandowski and Rodgers 2004, Meschi *et al.* 2010). While most physicians agree that dietary interventions should be encouraged after the development of a stone, consensus on the specifics of these changes have been lacking, with the exception of increasing hydration (Harvey *et al.* 1985, Curhan *et al.* 1993, Curhan *et al.* 1997a, Messa *et al.* 1997, Lemann 2002, Heller *et al.* 2003). Several different kinds of citrate-containing compounds have been used in the treatment of urolithiasis (Pak *et al.* 1985, Pak *et al.* 1986a, Pak *et al.* 1992a, Barcelo *et al.* 1993, Ogawa 1994 Sakhaee *et al.* 1994, Whalley *et al.* 1996, Schwille 1997) and the favorable effects thereof have been mentioned in section 1.1.4.

With adequate hydration, stones with a diameter < 5 mm will be spontaneously expelled from the renal system in ~ 90% of patients. However as the size of the stone increases, the likelihood of passage decreases and stones with a diameter of 1 cm have a 10% chance of being passed out without surgical intervention. Open surgical removal of stones is still an option for some patients (< 1%) (Matlaga and Assimos 2002). However over the years minimally invasive techniques, such as percutaneous nephrolithotomy (PCNL), Nephro-ureteroscopy (URS) and extracorporeal shockwave lithotripsy (ESWL) have revolutionized the management of urolithiasis. PCNL is the procedure where stones are removed through a small puncture made through the skin. URS is the removal of stones by fiberoptic nephron-ureteroscopes. ESWL is the most common of the aforementioned techniques and involves the use of mechanical shock waves to shatter stones into smaller fragments which are subsequently passed out of the body (Bataille *et al.* 2000, Coe *et al.* 2005, Samplaski *et al.* 2009, Buchholz *et al.* 2011, Cortes *et al.* 2011).

Even though highly effective, these surgical techniques also risk compromising renal function in some way. Complications that could arise include injury to renal blood vessels, infection, interstitial inflammation, severe hematuria, cellular damage and persistent renal fragments that may serve as a nidus for new stone formation (Schmidt *et al.* 2001, Pais and Assimos 2005, Samplaski *et al.* 2009).

The health care costs associated with the treatment of renal stone disease has risen considerably over the years (Trinchieri 2006). In the USA, this figure was estimated to be 2.5 – 3 billion dollars per annum (Chandoke 2005). Furthermore, it was reported that each new stone incidence in the UK costs the local health care system approximately £2000 (Robertson 2006).

Urolithiasis is becoming more prevalent and although pathogenesis is not completely understood, the risk of heat and climate has been well established, as mentioned in section 1.1.1. Recent estimates from computer models have predicted up to a 10% rise in the prevalence rate in the forthcoming half century due to the secondary effects of global warming, coinciding with a 25% increase in health-care expenditures (Romero *et al.* 2010, Fakheri and Goldfarb 2011). Taking into consideration the high frequency of stone recurrence, more cost-effective alternate methods of treatment and medical prophylaxis have become an option for stone patients.

In recent years, herbal remedies have been found to be useful in the treatment of urinary stone disease with fewer side-effects than contemporary Western medicine (Koide *et al.* 1995, Mok and Chau 2006, Butterweck *et al.* 2009). There remedies may therefore be suitable for long-term treatment. There are different schools of herbal medicines such as traditional Chinese medicine (TCM) and Ayurvedic medicine and these will be discussed in the following section.

1.4.2 Traditional Chinese Medicine (TCM)

The use of herbal treatment for kidney stone disease has been practiced in China and Hong Kong long before the introduction of Western medicine. This medicinal system has renewed popularity around the world due to its effectiveness in preventing many diseases and imbalances in the body. In 1999, the Chinese medicine ordinance implemented a regulation which allows the use, manufacture and trading of Chinese medicines in Hong Kong and China (cited by: Gohel *et al.* 2006) and today TCM practitioners are active in many Western countries.

This therapeutic system employs the use of different herbal preparations in various mixtures. The selected blend is dependent upon the patient's particular ailment. In contrast to western medicine, TCM does not give names to diseases but instead diagnoses the imbalance in the patient i.e. the deviation from a healthy balance (Ikegami *et al.* 2004, Qin *et al.* 2004, Jiang 2005). After physical examination, the symptoms of a patient are interpreted according to the ancient theories of 4 diseased states. The diagnosis is based significantly on intuition, experience and observational skills of the practitioner. Based on this, a combination of herbs is prescribed which is intended to assist the patient to return to a balanced state. Although the efficacy of TCM is suggested by years of clinical observations, there is little scientific evidence on their pharmacodynamics (Koide *et al.* 1995, Yasui *et al.* 1999, Ikegami *et al.* 2003, Miyaoka and Monga 2009).

Various herbs and combinations thereof have been successfully used in the treatment and prevention of urinary stone disease. This method of treatment covers the whole urinary system, including balancing of mineral electrolytes and the endocrine system (Qin *et al.* 2004). Herbal anti-lithogenic agents are employed to 'dissolve' stones or facilitate their passage through the

urinary system in order to prevent retention which can lead to further growth (Gohel *et al*, 2006). Herbal anti-lithics also induce diuresis to flush out crystalline deposits from the kidneys. An *in vitro* study of a herbal blend containing the traditional herbs Takusya (*Alismatis Rizoma*), Chorei (*Polyporous*), Bukuryo (*Hoelen*), Kasseki (*Talcum Crystallium*), Kinsensou (*Desmodii Herba*) and Kagosou (*Purunellae Spica*) revealed inhibitory effects on CaOx crystallization (Koide *et al.* 1995). The results of the study showed that with increasing concentrations of these 6 herbal extracts, the size of the calcium oxalate crystals formed, decreased (Koide *et al.* 1995).

In another *in vitro* study, the effects of 16 herbal extracts (including the 6 mentioned above) on calcium oxalate formation were tested. The two extracts showing the greatest potential for calcium oxalate crystallization inhibition, namely Takushya and Kagosou, were tested using an *in vivo* rat model. Of the two, Takushya showed a more significant inhibitory effect on CaOx crystallization (Koide *et al.* 1995). In addition to the aforementioned inhibitory effect, the administration of Takushya in rats induced a decrease in the expression of osteopontin, a stone matrix protein (Yasui *et al.* 1999). Osteopontin is strongly expressed in the kidneys of stone formers therefore down regulation of the expression of osteopontin is considered to be anti-lithogenic (Kohri *et al.* 1990). Chorei-to, a herbal mixture containing Takushya, Chorei, Bukuryo, Akyoh and Kasseki were tested in a rat model and exhibited stone prophylaxis (Yasui *et al.* 1999). In a clinical trial involving human subjects, Chorei-to was administered orally to patients who suffered from lower urinary tract problems. A total efficacy rate of 76% was achieved and no side-effects were observed (Horii *et al.* 1988).

Further *in vitro* studies have been carried out by Gohel *et al.* (2006) on the effect of *Folium pyrrosiae*, *Desmodium styracifolium*, *Pyrrosia Sheareri*, Dong Kui Zi and Siaoniaotong pills on calcium oxalate crystallization. These studies showed that all 5 plant extracts promote nucleation while inhibiting growth. Therefore they may be suitable for prophylaxis of urinary stones (Gohel *et al.* 2006). It is important to note that an increase in crystal nucleation is regarded as a favourable effect in the context of stone formation because it decreases urinary supersaturation (Kavanagh 1992). Thus small crystals are formed and expelled prior to them undergoing growth and aggregation. *Folium pyrrosiae* and *Desmodium styracifolium* are herbs of interest in this thesis.

1.4.3 Ayurvedic medicine

Ayurveda is a Sankrit term meaning 'the knowledge of life span'. It is an ancient medicinal system from India that originated thousands of years ago. The fundamental principles of Ayurvedic medicine can be traced to the *Vedas*, the oldest books of Indian knowledge. It is considered to be a form of alternative medicine in the West and encompasses different methods of treatment such as the use of herbs, massage, meditation and yoga (Mitra *et al.* 1998, Tomlinson 2002, Kieley *et al.* 2008, Patwardhan *et al.* 2009). Ayurvedic medicine is also based on the maintenance of health through the balance of the three essential humors (bodily fluids): wind, gall and mucus. Imbalances in these humors, also referred to as *dosas*, manifest themselves as diseases in the body. Treatment is based on restoring homeostasis of these factors (Kieley *et al.* 2008). As with TCM, herbs are prescribed in blends as they work synergistically. Most medicines for urinary calculi disease have a wide range of action, including: analgesic, anti-inflammatory, anti-microbial, diuretic, antispasmodic properties, and anticalcifying activities. Some of the most popular and effective Ayurvedic herbs for treating renal calculi are: *Tribulus terrestris* (Gokshura), *Arctostaphylos* (Bearberry Uva-Ursi), *Phyllanthus niruri* (Bahupatra), *Orthosiphon stamineus* (Java Tea) and *Orthosiphon grandiflorus* (Joshi *et al.* 2005, Kieley *et al.* 2008). *Phyllanthus niruri* and *Orthosiphon stamineus* are herbs that will be investigated in this thesis.

Tribulus terrestris is one of the most commonly administered Ayurvedic medicines for renal problems. Its primary use is as a diuretic (Joshi *et al.* 2005). *In vitro* studies in which COM crystals were grown by a double diffusion gel growth technique, showed a significant inhibition of crystal growth brought about by an aqueous extract of *Tribulus terrestris* (Joshi *et al.* 2005). Using a rat-model, the mechanism by which *Tribulus terrestris* prevents nephrolithiasis was found to be by decreasing the excretion of urinary oxalate by inducing changes in hepatic oxalate synthesizing enzymes: glycolate oxidase (GAO) and glycolate dehydrogenase (GAD) (Joshi *et al.* 2005). Suppression of these two enzymes as well as renal lactate dehydrogenase (LDH), a promoter of high urinary oxalate levels, was noted in sodium glycolate-fed rats that were administered with *Tribulus terrestris* (Sangeeta *et al.* 1994).

1.5.4 Herbal remedies for urolithiasis

As mentioned, herbal remedies are increasingly being considered as a suitable long-term treatment for renal dysfunction due to a reduced likelihood of side-effects and no known toxicity (Atmani *et al.* 2000, Gohel *et al.* 2006). There are not many published papers describing these effects however strong anecdotal evidence exists in support of their existence. Hence rigorous scientific analyses of these anecdotal observations are warranted. A few plants which are indigenous to different parts of the world purported to be effective in the treatment of urolithiasis, have been selected for investigation in the present project. These plants are introduced below.

***Folium Pyrrosiae* (Shi Wei)**

Folium pyrrosiae, an evergreen plant that grows throughout China, is the principal plant of interest in this thesis and is most prominently recommended by TCM practioners for the treatment of urinary stone disease. According to the principles of TCM, the leaves of *Folium pyrrosiae* have bitter, sweet and mildly cold properties and are used in treating lung and bladder disorders (Gohel *et al.* 2006). Its main functions are to clear damp (one of the 6 influences causing disharmony to the body) and expel phlegm from the body (Ma *et al.* 2003). In relation to urinary stone disorders, *Folium pyrrosiae* acts as a diuretic and antibacterial. It relieves strangury (painful drop by drop release of urine) and has demonstrated its potential as being a prophylaxis of stones by promoting nucleation of CaOx crystals and inhibiting crystal aggregation and growth in vitro in synthetic urine (Gohel *et al.* 2006). The main chemical ingredients in the leaves of the plant are tannins, saponins, anthraquinones and chlorogenic acid (Li and Tong 1992).

***Desmodium styracifolium* (Jin Qian Cao)**

The *Desmodium* genus grows widely in the tropical and sub-tropical regions of China. *Desmodium styracifolium* is another important TCM plant used in the treatment of urinary stone disease (Gohel *et al.* 2006). It reduces heat and swelling and induces diuresis and acidification of urine (Gohel *et al.* 2006). *In vitro* studies on *Desmodium styracifolium* have shown a significant increase in nucleation of CaOx (Hirayama *et al.* 1993, Gohel *et al.* 2006) which thereby

decreases the probability of stone formation. The chemically active ingredients in *Desmodium styracifolium* are polysaccharides, triterpenoids and flavonoids (Ming *et al.* 2007).

***Hylocereus trigonus* (Odyvato)**

Hylocereus trigonus belongs to the cacti family. It grows prominently in equatorial regions, especially in the Carribean and Puerto Rico but is also found in Madagascar. It is a well-known folk medicine and is commonly called Odyvato. The dried aerial parts of the plant are brewed as a drink and it is used to treat kidney stone ailments. Even though Odyvato is administered to stone patients routinely in Madagascar, there is no published data to support its mechanisms of action again kidney stone disease (*Private communication:* Professor Philippe Rasoanaivo, *Institut Malgache de Recherches Appliquées*, Madagascar).

Phyllanthus niruri

Phyllanthus niruri belongs to the family of *Phyllanthaceae* and grows worldwide in tropical and subtropical regions. It has been used in Ayurvedic and Brazilian folk medicine for the treatment of urolithiasis to control pain and eliminate stones. Several studies, including *in vitro* and *in vivo* (human and rat model), have been carried out on *Phyllanthus niruri* and its potency has been demonstrated in its ability to inhibit CaOx aggregation and growth (Freitas *et al.* 2002, Barros *et al.* 2003, Barros *et al.* 2006). Furthermore its ability to normalize elevated urinary calcium levels in calcium stone forming patients has been demonstrated (Micali *et al.* 2005). This herb is commercially available as Uriston® in western countries. Regular treatment with *Phyllanthus niruri* post ESWL in stone patients was found to significantly decrease stone recurrence (Micali *et al.* 2005). Many chemical components of *Phyllanthus niruri* have been identified such as alkaloids, tannins, lignans, phenols, steroids, flavanoids and triterpenes (Barros *et al.* 2003).

Orthosiphon stamineus

Also known as Java tea and 'Cat's Whiskers', *Orthosiphon stamineus* is a native plant of South Asia and belongs to the family *Lamiaceae*. It is a well-known traditional Indonesian and Ayurvedic medicine that is used in the treatment of kidney stone disease and urinary tract infections (Hirayama *et al.* 1993, Adam *et al.* 2009). Arafat *et al.* (2008) demonstrated the diuretic and hypouricemic (low serum uric acid) effects of *Orthosiphon stamineus* using a rat

model. Dharmaraj *et al.* (2008) reported the inhibitory effect of *Orthosiphon stamineus* on CaOx crystal growth by conducting *in vitro* studies on a 50% methanol extract of the plant material. It is also known to act as a diuretic and to reduce inflammation (Dharmaraj *et al.* 2006). The plant has been widely studied and the leaf extract has revealed the following constituents: terpenoids, polyphenols, sterols and caffeic acid derivates (Dharmaraj *et al.* 2006, Arafat *et al.* 2008).

Cystone®

Cystone® is a polyherbal Ayurvedic preparation produced by the Himalaya Drug Company. This herbal formulation is used in the treatment of urolithiasis. Its constituent plants are: *Didymocarpus pedicellata*, *Saxifraga ligulata*, *Rubia cordifolia*, *Cyperus scariosus*, *Achyranthes aspera*, *Onosma bracteatum*, *Vernonea cinerea* and *Tribulus terrestris* (Jethi *et al.* 1983, Mitra *et al.* 1998, Erickson *et al.* 2011). *In vitro* studies by Jethi *et al.* (1983) showed demineralization (i.e. dissolution) of the crystal matrix and inhibition of deposition of calcium and phosphate ions. Cystone® also induced a reduction in cisplatin induced renal-and nephrotoxicity as shown by rat models (Rao and Rao 1998, Rao *et al.* 1999)**.** In another study, Cystone demonstrated the ability to aid expulsion of stones in patients (Kumaran and Patki 2011). *Didymocarpus pedicellata*, the main component of Cystone® tablets, posseses a high concentration of polyphenolics and antioxidants (Kaur *et al.* 2007).

1.5 Urolithiasis in South Africa

As stated previously, in South Africa the occurrence of stones amongst the black population is extremely rare (< 1%) while in the white population stone occurrence is similar to the incidence rate in the Western world (~ 15%) (Modlin 1967, Beukes *et al.* 1987, Meyers 1994, Whalley *et al.* 1998).

Studies of the urine composition of these two population groups have revealed interesting results. It has been reported that blacks actually have a lower urinary citrate and higher urinary oxalate concentrations which would cause them to be **more** susceptible to stone formation (Modlin 1967, Whalley *et al.* 1998, Lewandowski and Rodgers 2004). However, this does not happen and is somewhat perplexing. On the other hand, urinary calcium and phosphate excretions have been found to be significantly lower in black subjects compared to white (Modlin 1967, Whalley *et al.* 1998, Lewandowski *et al.* 2001, Lewandowski and Rodgers 2002). Further interesting results were obtained from studies on urinary pH where this parameter was found to be lower in black subjects (Modlin 1967, Lewandowski *et al.* 2001, Rodgers *et al.* 2005). However all these values **lie within the normal range**. Thus although the lower excretions of these two parameters may explain the lower incidence in the black population, it cannot account for its near absence in the black group. Therefore urine composition alone cannot account for the rarity of stones in the black population.

In order to elucidate the differences in stone frequency in the two race groups, several studies have been undertaken by the Kidney Stone Research Laboratory (KSRL) at the University of Cape Town where the renal handling of different dietary and supplemental challenges have been investigated.

Lewandowski and Rodgers (2004a) investigated individual effects of 5 different dietary or supplemental challenges (high dietary calcium; calcium, vitamin B_6, L-glutamine and L-cysteine supplementation) on urinary risk factors of CaOx kidney stone formation. None of these protocols altered any biochemical of physiochemical factors in black subjects, however the following significant changes were found in white subjects: dietary calcium increased urinary

potassium and decreased relative supersaturation of brushite; supplemental calcium decreased the Tiselius Risk Index; vitamin B_6 decreased urinary calcium, urinary phosphate and relative supersaturation of brushite; L-glutamine decreased the relative supersaturation of CaOx; and L-cysteine decreased urinary calcium and TRI. Since none of these factors was detected in blacks, these workers speculated that renal or gastrointestinal homeostatic adjustment occurs in this group (Lewandowski and Rodgers 2004a).

In another supplementation study, evening primrose oil was administered to black and white subjects and the effects of urinary risk factors were investigated. The principal ingredient in this supplement is γ-linolenic acid, an ω-6 polyunsaturated fatty acid. Urinary citrate increased in both groups and urinary calcium and TRI decreased in both groups. A decrease in urinary oxalate was detected in the black group but not in the white group indicating the sensitivity of the former group to this supplementation (Rodgers *et al.* 2009a).

Further investigations have been carried out on carbohydrate (glucose, sorbitol and xylitol) and oxalate-containing dietary agents (rhubarb, spinach and an aqueous solution of sodium oxalate) (Rodgers *et al.* 2009b). These agents were selected as dietary surveys revealed that the consumption of these potentially lithogenic substances were higher in the black population group. The results showed that these agents initiated more significant urinary changes in black subjects compared to whites. The only changes measured in white subjects was upon consumption of sorbitol which induced an increase in urinary oxalate and a decrease in urinary phosphate. However in black subjects, the following urinary changes were detected: an intake of glucose resulted in a decrease in phosphate, an intake of sorbitol also resulted in a decrease of phosphate, whereas an intake of xylitol resulted in an increase of oxalate. It has been proposed that decreases in urinary phosphate are a consequence of reduced renal filtered load of phosphate due to entry of phosphate into cells after glucose or sorbitol ingestion. Whereas increased urinary oxalate following sorbitol and xylitol occurs via metabolic pathways which are governed by certain enzymes that convert the two sugars into oxalate. The activity of the enzyme aldolase which was found to vary in the two groups, hence differences in the amounts measured.

Furthermore, the black population is able to handle dietary oxalate in a more efficacious way compared to their white counterparts (Lewandowski *et al.* 2001). In additional studies, it was found that even when black subjects consumed a greater amount of dietary oxalate than white subjects, urinary oxalate levels were not significantly different. In addition, these studies have revealed that black subjects also have significantly more oxalate-degrading bacteria that have greater capabilities than those isolated from white subjects (Lewandowski *et al.* 2001, Rodgers *et al.* 2002, Lewandowski *et al.* 2004, Lewandowski *et al.* 2005).

These studies have shown that the renal response and therefore renal handling mechanisms in the subjects from the two groups is different and it is the handling of supplemental challenges that is of interest in the present study.

Investigation of the role of urinary proteins in the two population groups was another attempt at trying to understand the anomaly of stone incidence that exists between South Africa's black and white population groups. Analysis of THM and UPTF1 (explained in section 1.2.5), has suggested the urine composition in black subjects enhances a superior inhibitory activity of these proteins (Craig *et al.* 1999, Webber *et al.* 2002). The *N*-linked glycans on UPTF1 isolated from black subjects was found to have a significantly greater proportion of disialylated fragments compared to those found in white subjects (Webber *et al.* 2004). Bikunin, a plasma protease inhibitor, isolated from matrix extract proteins was found in greater quantities black subjects compared to that in white subjects. In further studies, OPN isolated from the urine of black subjects has demonstrated greater inhibition of CaOx aggregation and deposition, and decreased promotion of nucleation than OPN derived from the urine of the whites (Deppa *et al.* 2005). Whereas urinary albumin isolated from the two race groups was found to have different molecular and structural differences, hence different activity (Rodgers *et al.* 2006b). These observed inhibitory properties of proteins in the black population group may be key role-players in their immunity to stone disease.

Although many studies have been performed at the University of Cape Town, no single explanation has emerged regarding the difference in stone prevalence between blacks and whites, though differences have been reported to be contributory.

1.6 Objectives

There is minimal evidence that any herbal remedy is actually effective in preventing stone recurrence. Furthermore, in those few cases where a remedy has been effective, there is no scientific evidence as to how it may work. Although it could be via an alteration in the crystallization processes or crystal-cell binding, the role of these phenomena is still uncertain. It is equally likely that herbal remedies might work via completely different mechanisms, such as the formation of Randall's plaque. Thus a scientific study that investigates the possible efficacy of herbal remedies and the mechanisms by which they act is warranted. The present thesis aims to address some of these.

The first principal objective of the project described in this thesis was to develop and establish a model for the *in vitro* and *in vivo* investigation of traditional herbal preparations which have been previously advocated for the possible treatment of urolithiasis and to implement the model in the investigation of several such preparations.

The second principal objective was to investigate the renal response of subjects from South Africa's black and white population groups to the administration of a herbal preparation which has been widely used for the treatment of kidney stone disease in the Tradition Chinese Medicinal system, with a view of gaining insights into understanding why the incidence of this disease in the former group is extremely rare.

In order to achieve these main objectives, the following specific aims were defined:

(i) Investigate the independent *in vitro* effects of several traditional herbal preparations on CaOx crystallization characteristics in a synthetic urine

(ii) Investigate the independent *in vitro* effects of several traditional herbal preparations on CaOx crystallization characteristics in real urine derived from healthy black and white South Africa subjects

(iii) Investigate the independent *in vitro* effects of several traditional herbal preparations on the binding of urinary and inorganic CaOx crystals to animal renal cells

(iv) Investigate the *in vivo* renal response with respect to urinary composition and supersaturation, to the administration of *Folium pyrrosiae* in black and white South African subjects

(v) Characterize the chemical composition and structure of *Folium pyrrosiae* with a view to identifying the active ingredient and, in so doing, establish a protocol for characterization of other such herbal preparations

General methods

This chapter provides details on the general laboratory procedures which were used to achieve the objectives of this thesis. It begins by giving details of how the plant extracts under investigation were obtained, followed by descriptions of the preparation of synthetic urine (SU) and the collection and treatment of 24 hour urine samples. Details on the measurement of the CaOx *metastable limit* (MSL) are given. Thereafter, experiments to determine: *particle size-volume distribution* (PSD); *^{14}C-oxalate deposition kinetics*; and inhibition kinetics of *crystal nucleation, aggregation* and *growth* are described. Details on examination of crystalluria by *scanning electron microscopy* (SEM) and calculation of the various *physicochemical risk indices* are also presented.

2.1 Origin of plant extracts

Folium pyrrosiae and *Desmodium styracifolium* were purchased individually under the brand name *Nong's Powder*, from PuraPharm pharmaceutical company, Hong Kong. *Hylocerus trigonus* was obtained from the Institut Malgache de Recherches Alliquées, Madagascar. It is commercially available in Madagascar as *Masy Odivato*. *Phyllanthus niruri* (a product of Peru) was purchased from Raintree Nutrition, Nevada, USA. *Orthosiphon stamineus* was purchased as *Java Tea* from Midas Herbs, Indonesia. Cystone®, manufactured by the Himalaya Drug Company, was obtained from an Ayurvedic practitioner, Dr Rajen Coopan (Durban, South Africa).

2.2 Preparation of synthetic urine

SU was prepared according to the method of Walton *et al.* (2005) for *in vitro* crystallization experiments. A 1 litre solution was constituted by the addition of the following salts at the given concentrations: 0.320 M NaCl (*Orion Chemicals*), 0.050 M NaH_2PO_4 (*Merck*), 0.00652 M $MgCl_2$ (*Riedel-de Haën*), 0.164 M KCl (*Merck*), 0.00434 M K_3Citrate (*Merck*), 0.0438 M $(NH_4)_2SO_4$ (*Merck*) and 0.007 M NH_4Cl (*Merck*). During preparation of SU, salts were added in the above sequence and care was taken to ensure that each salt was dissolved before adding the

next. The pH of the mixture was adjusted to 6.0 by the addition of 5 M NaOH. The solutions were stored at 4 ˚C for a maximum of 4 days after preparation and filtered (0.22 μm) immediately before use to remove any particulate matter. Solutions of 0.005 M NaOx (*Chemical Enterprises*) and 0.120 M $CaCl_2$ (*Merck*) were prepared separately and the following volumes were added per liter SU: 2.86 mL NaOx and 17.8 mL $CaCl_2$.

2.3 Urine collection and treatment

For experiments in real urine, 24 hour samples were collected in plastic bottles without preservative. Extensive details concerning aspects of urine collection in the various experiments are described for those studies elsewhere in this thesis, in sections which have been dedicated. Black and white male subjects aged between 18 and 32 years old, with no prior history of kidney stone disease, were recruited from the cohort of students from the University of Cape Town. Subjects were asked to restrict their diet with respect to oxalate rich foods. Urine collections that tested positive for the presence of haematuria or nitrites using urinalysis test strips (*Boehringer Mannheim, Germany*) were excluded from the study. Urine was pre-filtered through a 0.75 μm filter followed by 0.45 μm nitrocellulose filter paper before use to remove cellular debris.

Biochemical urine measurements

An aliquot of each urine sample was retained for urinalysis. Urinary pH and volume were routinely measured (*pH meter 211, Hanna Instruments, USA*). Sodium, potassium, calcium and magnesium were analyzed by atomic absorption spectroscopy (*Varian 1275, Australia*) (Willis 1961, Trudeau and Freier 1967, Fernandez and Kahn 1971). Oxalate and citrate concentrations were determined by enzymatic assays. Analytical kits were employed in the measurement of oxalate (*Trinity Biotech, Ireland*) (Chiriboga 1963) and citrate (*Boehringer Mannheim, Germany*) (Gruber and Mollering 1966). Inorganic phosphate was measured using ammonium molybdate (Dryer and Routh 1963), creatinine was measured using picric acid (Rock *et al.* 1986) and uric acid was measured using uricase (Fossati *et al.* 1980). Chloride determination was carried out by means of a chloride sensitive electrode.

2.4 CaOx metastable limit (MSL)

CaOx MSL is a measure of the ability of urine to resist spontaneous nucleation and hence crystallization. This method described by Ryall *et al.* (1985, 1995) was followed. Urine samples were prepared by successively filtering through a 0.75 µm pre-filter (*Macherey-Nagel, Germany*) followed by a 0.45 µm nitrocellulose membrane (*Sartorius AG, Germany*). Aliquots of 10 mL of filtered urine were pipetted into Coulter cups and incubated at 37 ˚C in a circulating water bath at 100 rpm (*Labcon, Johannesburg*). To each aliquot, 100 µL of progressively increasing concentrations of sodium oxalate (Na_2Ox; 15 mmol/L to 195 mmol/L) were added. Dosing was performed at 2 minute intervals. After incubation for a total of 30 minutes for each sample, particle numbers and volumes were measured using a Coulter Multisizer II (*Coulter Electronics Ltd., England*) fitted with a 140 µm orifice (2.8 – 90.0 µm particle size range). The MSL corresponds to the minimum Na_2Ox concentration needed to induce spontaneous crystallization (denoted by the pink dot). It is determined by plotting particle size vs Na_2Ox concentration as shown in Figure 2.1.

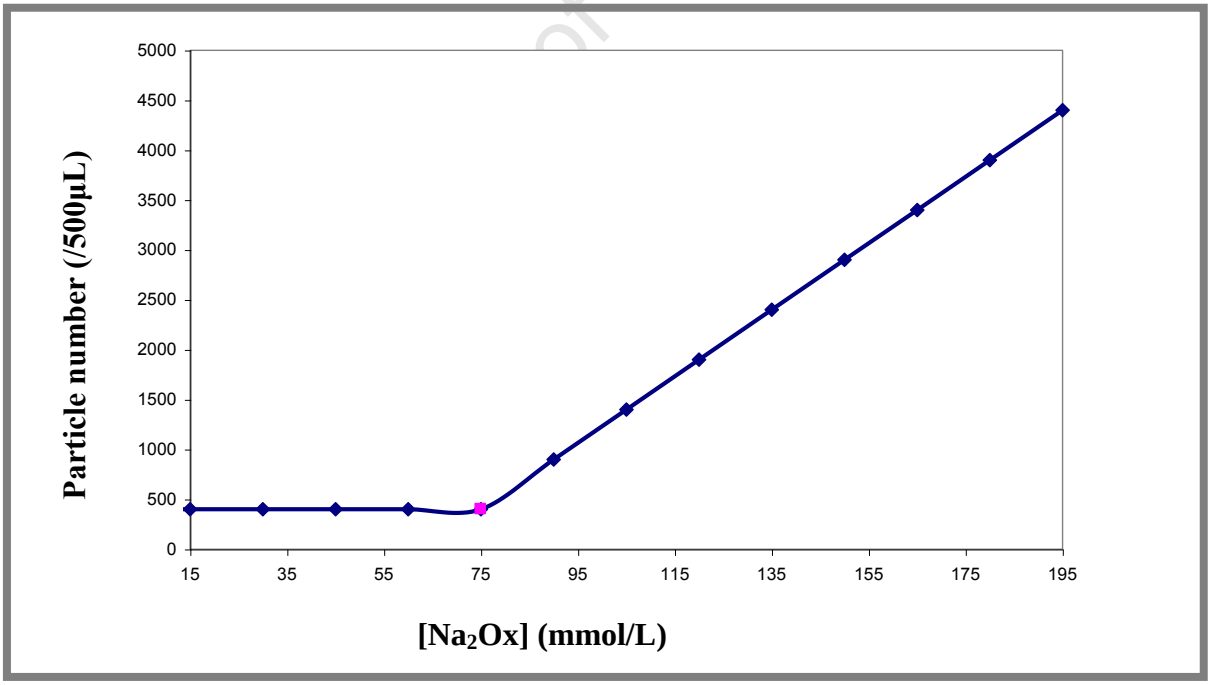


Figure 2.1: A typical plot to determine the metastable limit

2.5 CaOx particle size-volume distribution

CaOx particle size-volume distributions were determined according to the method described by Ryall *et al.* (1995). Each urine sample (30 mL) was incubated at 37 ˚C in a shaking water bath at 100 rpm (*Labcon, Johannesburg*). Once the temperature of the urine had equilibrated, an aliquot of 10% (v/v) Na_2Ox corresponding to 30 mmol/L above the MSL, was added to induce crystallization.

After a 2 hour incubation period, the total volume of the particles precipitated and mean particle size were determined using a Coulter Multisizer II (*Coulter Electronics Ltd., England*). Graphs of particle volume vs particle size were plotted. The mode of the curve, determined using *Software R* (Azzalini and Capitanio 1999), is a representation of the average size of precipitated particles, whereas the total volume is calculated by integration of the curve. A typical volume vs size graph is given in Figure 2.2.

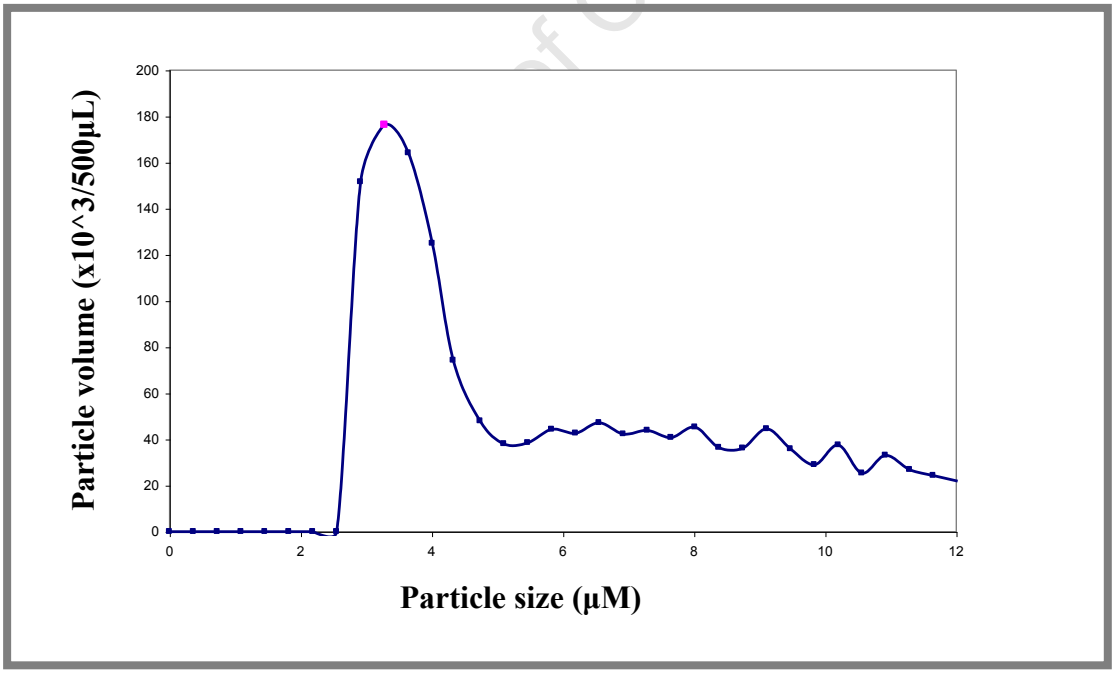


Figure 2.2: A typical plot of particle-size volume distribution

2.6 Scanning electron microscopy (SEM)

Urine samples were prepared as described in the CaOx particle size-volume experiment. After the 2 hour incubation period, 2 mL of the dosed urine was filtered (0.22 μm) and the filter paper was dried at room temperature. Once dried, the filter paper was mounted on aluminium stubs using *Sticky Tabs* (*ProSciTech, Thailand*). Crystals were viewed using a *Leica* S440 scanning electron microscope (*Leica Cambridge Ltd., England*) operating at an accelerating voltage of 10 kV, a working distance of 10-15 mm and a probe current of 20-30 pA. The magnification at which stubs were viewed varied for different studies. However micrographs for a particular experiment were recorded at the same magnification so that valid comparisons could be made.

2.7 ^{14}C-oxalate deposition kinetics

There are known limitations of the Coulter Counter in determining the volume of particles i.e. the inability to recognize crystal aggregates and to measure crystal sizes outside a specific range. Therefore, to determine particle-volume as an accurate reflection of an increase in crystal mass, ^{14}C-oxalate was used in experiments where a decrease in soluble radioactivity correlates to precipitation of CaOx (Ryall *et al.* 1995).

The method described by Ryall *et al.* 1995 was used for this experiment. Urine (30 mL) was pipetted into soda-lime flasks (to prevent crystals from sticking to the sides) and incubated in a shaking water bath (100 rpm) at 37 ˚C. At equilibrium, 3.125 μCi ^{14}C-oxalic acid (*NEN, Boston, USA*) per 100 mL of urine was added. Crystallization was then induced by the addition of Na_2Ox (10 % v/v) at a concentration of 30 mmol/L above the previously determined MSL and the total incubation time was 120 minutes. At 30 minutes intervals, starting with the first reading at 0 minutes, a 2.5 mL aliquot was removed and filtered into concentrated hydrochloric acid (10 % v/v) in order to quench the reaction so that no further crystallization could occur. 1 mL of this acidified urine was added to two aliquots of 10 mL scintillation fluid (*Zinsser Analytic, Frankfurt*). ^{14}C was counted using a scintillation counter (*Beckmann LS 5000TD Scintillation Counter*), for 10 minutes.

The percentage of precipitated ^{14}C-oxalate was determined using the following equation (Doyle *et al.* 1995):

% of precipitated ^{14}C-oxalate = 100 – 100 x (cpm at x minutes/cpm at 0 minutes)

Graphs of percentage precipitated ^{14}C-oxalate were then plotted as a function of time. The following experimental systems were investigated: (1) filtered human urine, and (2) filtered human urine dosed with plant extracts. The activity of the plant extracts in filtered human urine were monitored by comparison with the control (undosed filtered human urine).

2.8 Crystallization experiments

2.8.1 Preparation and characterization of COM crystals

Pure COM crystals were prepared for the crystal growth kinetics assay (section 2.8.3). The method described by Pak *et al.* (1975) was followed. Using two line feeds, equal volumes of 0.010 M NaOx and 0.010 M $CaCl_2$ were mixed at a constant rate of 1 mL/min using a peristaltic pump with stirring. The mixture was allowed to stir for 7 days at 4 ˚C after which the crystals were collected by filtration (0.22 μm). The crystals were dried in an oven at 37 ˚C for approximately 1 hour. X-ray powder diffraction (XRD) analysis, described below, was used to confirm the presence of COM.

X-Ray powder diffraction

The x-ray powder diffraction pattern was determined using a Phillips PW1050/25 goniometer with a nickel filtered Cu-K α radiation source (λ = 1.5418 Å) produced at 40 kV and 25 mA. The crystals were ground with a mortar and pestle to produce a homogenous powder of constant particle size. The sample was placed in an aluminium holder. The intensity of the reflected x-rays as a function of the Bragg angle (2θ) was recorded between 12-40˚ for each sample. The x-ray diffraction pattern obtained for CaOx crystals is shown in Figure 2.3. The angles corresponding to the maximum peaks of the scan were converted to their equivalent d-spacings

using a standard table for Cu-K α radiation based on the Bragg Equation (Parrish and Mack 1963). These d-spacings were then compared with standard reference values for COM crystals (Sutor and Scheidt 1968). Table 2.1 confirms that the crystals are COM.

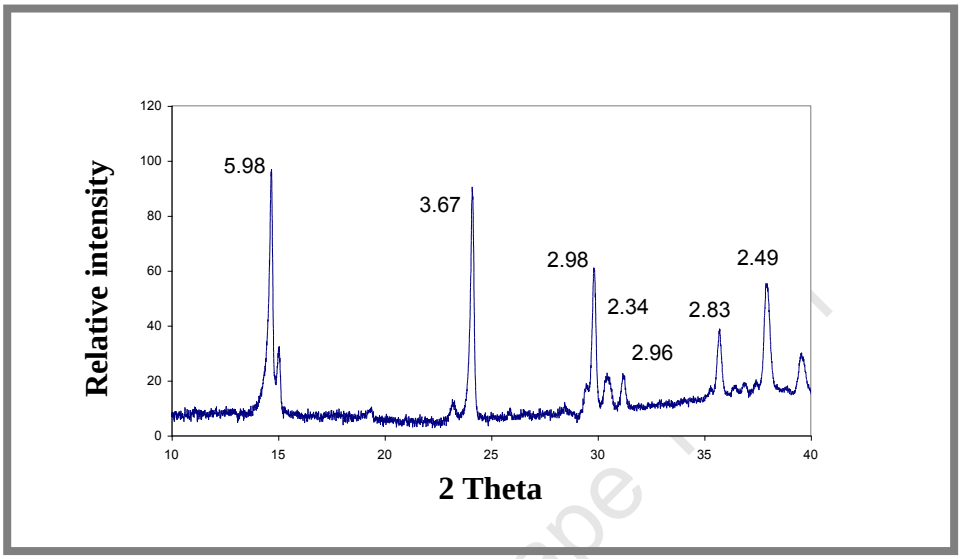


Figure 2.3: XRD pattern of CaOx crystals prepared by the method of Pak *et al.* (1975)

Table 2.1: Interplanar spacings and relative intensities of powder patterns on COM

Standard values for COM		**Present study**
d-spacing (Å)	***Relative intensity***	***d-spacing (Å)***
5.93	100	5.98
5.79	25	
4.64	7	
4.52	6	
3.78 }		
3.76 }	13	
3.65	100	3.67
3.00	10	
2.97	46	2.98
2.91	12	2.96
2.89	10	
2.84	14	2.83
2.51	2	
2.48	30	2.49
2.41	5	
2.37	2	
2.34	90	2.33

2.8.2 Simultaneous measurement of CaOx crystal nucleation and aggregation kinetics

The spectroscopic method developed by Hess *et al.* (1995) was used to simultaneously determine the inhibition kinetics of CaOx nucleation and aggregation. This simple batch (discontinuous) system is widely used in crystallization research. It involves the mixing of two solutions (calcium and oxalate) which when combined produce a metastable supersaturated solution of CaOx. Once crystallization is induced, supersaturation will reduce as calcium and oxalate are removed from the solution. The decay of supersaturation or change in turbidity is followed continuously.

A graph of absorbance vs time is plotted and the gradient of this graph represents an increase in the rate of particle number i.e. crystal nucleation. When equilibrium (saturation) is reached, crystals can neither nucleate nor grow. A progressive decrease in turbidity is observed reflecting the rate of decrease in particle number due to aggregation then occurs (Hess *et al.* 1995, Hess and Kok 1996).

Stock solutions of 8.5 mmol/L calcium chloride ($CaCl_2$) (containing 200 mmol/L sodium chloride and 10 mmol/L sodium acetate) and 1.0 mmol/L Na_2Ox (containing 200 mmol/L sodium chloride and 10 mmol/L sodium acetate), were prepared. The pH was adjusted to 5.7 and the solutions were filtered through a 0.22 μm membrane.

The stock solutions of $CaCl_2$ and Na_2Ox were allowed to equilibrate to 37 ˚C in a shaking water bath at 100 rpm (*Labcon, Johannesburg*). To a glass cuvette, 1 mL $CaCl_2$ solution was added, and placed in a spectrometer (*Specord 40, Analytikjena, Germany*). Thereafter, 1 mL Na_2Ox was added give final assay concentrations of 4.25 mmol/L calcium and 0.5 mmol/L oxalate. The temperature in the cuvette was maintained at 37 ˚C and stirred at 500 rpm (*Analytikjena, Germany*) throughout the experiment. The absorbance at 620 nm was measured every 0.5 second for 60 minutes. The maximum increasing slope of OD_{620nm} vs time is measure of crystal nucleation kinetics, termed $\mathbf{S_N}$. Once equilibrium was achieved, the decrease in the slope of OD_{620nm} vs time represents crystal aggregation kinetics, termed $\mathbf{S_A}$. A typical nucleation plot is given in Figure 2.4.

The following experimental systems were investigated: (1) SU, (2) SU dosed with plant extracts, (3) filtered human urine, and (4) filtered human urine dosed with plant extracts.

Percentage inhibition of nucleation and aggregation were calculated using the following equation, where $\mathbf{T_s}$ = turbidity of test sample, and $\mathbf{T_c}$ = turbidity of control sample:

percentage inhibition of nucleation (% I_N) = $100(1 - T_s/T_c)$

percentage inhibition of aggregation (% I_A) = $100(1 - T_s/T_c)$

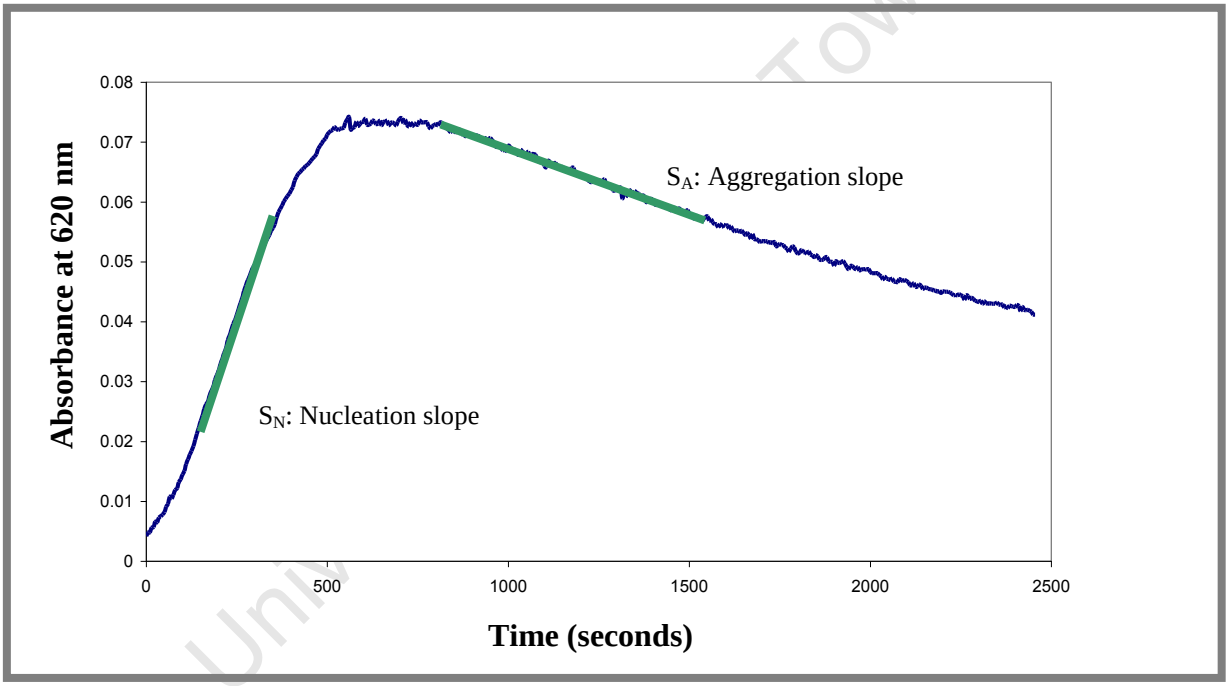


Figure 2.4: A typical plot of absorbance vs. time for the simultaneous determination of CaOx nucleation and aggregation kinetics

2.8.3 CaOx crystal growth kinetics

Crystal growth kinetics were determined according to the method adapted by Webber *et al.* (2007). This is an example of a seeded batch system and is performed by setting up a metastable saturated solution and introducing seed crystals which then grow. When equilibrium is reached, crystal growth will be represented by the kinetics of supersaturation decay and in this case, by the change in turbidity.

A seed crystal slurry was prepared by suspending COM crystals (prepared as described previously) in Tris buffer at a concentration of 16 mg/mL. The slurry was allowed to equilibrate overnight with fast stirring. Tris buffer was prepared by mixing 10 mM Tris-(hydroxymethyl)-aminomethane with 90 mM NaCl. The pH of the solution was adjusted to 7.2 and the buffer was filtered (0.22 μm) prior to use.

A metastable solution of CaOx, consisting of $CaCl_2$ (0.5 mM) and Na_2Ox (0.5 mM), was made up in Tris buffer. The metastable CaOx solution was maintained at room temperature with fast stirring. Care was taken to ensure that the solution did not turn cloudy while being stirred during the experimental period. In cases where cloudiness appeared, fresh solutions were made up. A 2 mL aliquot of the metastable CaOx solution was transferred to a quartz cuvette and placed in a spectrophotometer (*Specord 40, Analytikjena, Germany*) and stirred at 500 rpm (*Analytikjena, Germany*). The crystal seed slurry (50 μL) was added to induce growth. OD_{214nm} was recorded every second for a period of 15 minutes.

The following experimental systems were investigated: (1) SU, (2) SU dosed with plant extracts, (3) filtered human urine, and (4) filtered human urine dosed with plant extracts.

For experiments in SU, the ratio used was, 1600 μL metastable CaOx solution: 400 μL urine (SU): 50 μL COM slurry. For real urine samples, the ratio used in order to get detectable crystals was 1990 μL metastable CaOx solution: 10 μL urine (SU): 50 μL COM slurry (Hess *et al.* 1989). The inhibition of crystal growth (I_G) was determined by plotting a graph of absorbance (214 nm)

vs time (seconds) and calculating the slope of the linear decreasing region and using the formula given below. A typical plot is shown in Figure 2.5.

Inhibition of growth (I_G) = $[(A_C - A_T)/A_C] \times 100$

$A_C = \Delta_{absorbance}/\Delta_{time}$ (of the control)

$A_T = \Delta_{absorbance}/\Delta_{time}$ (of the test)

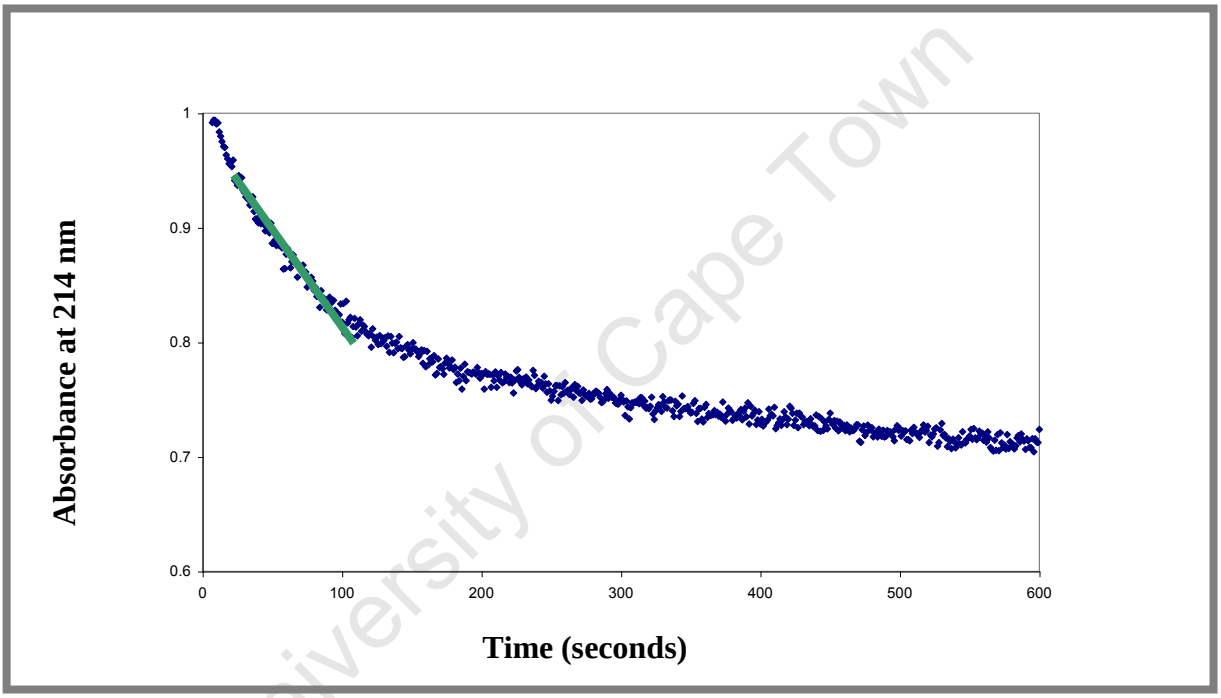


Figure 2.5: A typical plot of absorbance vs. time for the determination of CaOx growth

2.9 Mixed suspension mixed product removal (MSMPR) crystallizer

Renal stone formation is a result of a series of crystallization processes which occur within the kidney. A continuous flow of supersaturated urine passes through the renal system. In nephrolithiasis research, the MSMPR crystallizer is used to model the continuous flow system of the kidney. All experiments were carried out at 37 ºC in a constant temperature chamber, and therefore can be considered a biological analog of the renal system (Miller *et al.* 1977, Nishio *et al.* 1991, Kavanagh 1992, Bretherton and Rodgers 1998).

A simple schematic of the MSMPR system is shown in Figure 2.6. It consists of a peristaltic pump (*Watson Marlow, 205U*) that was used to control the flows of the feed and effluent streams. The temperature was maintained constant by circulating thermostatic water through the corresponding external water bath of the MSMPR chambers.

In order to induce crystallization in the MSMPR, the following solutions were continuously fed through the system: SU, 150 mM calcium chloride ($CaCl_2$) and 30 mM sodium oxalate ($Na_2C_2O_4$). The SU, with ion components corresponding to urine in the distal part of the collecting duct, was prepared according to Walton *et al.* as described in section 2.2. A constant volume of 20 mL was maintained in the MSMPR chamber. This was achieved by feeding the system with SU at a flow rate of 2.3 mL/minute and $CaCl_2$ and $Na_2C_2O_4$ at flow rates of 0.1 mL/min. The effluent stream rate was 2.5 mL/min which equalled the feed rate (2.3 mL/min + 0.1 mL/min + 0.1 mL/min). The composition of the feed stream was 92 % AU, 4 % $CaCl_2$ and 4 % $Na_2C_2O_4$.

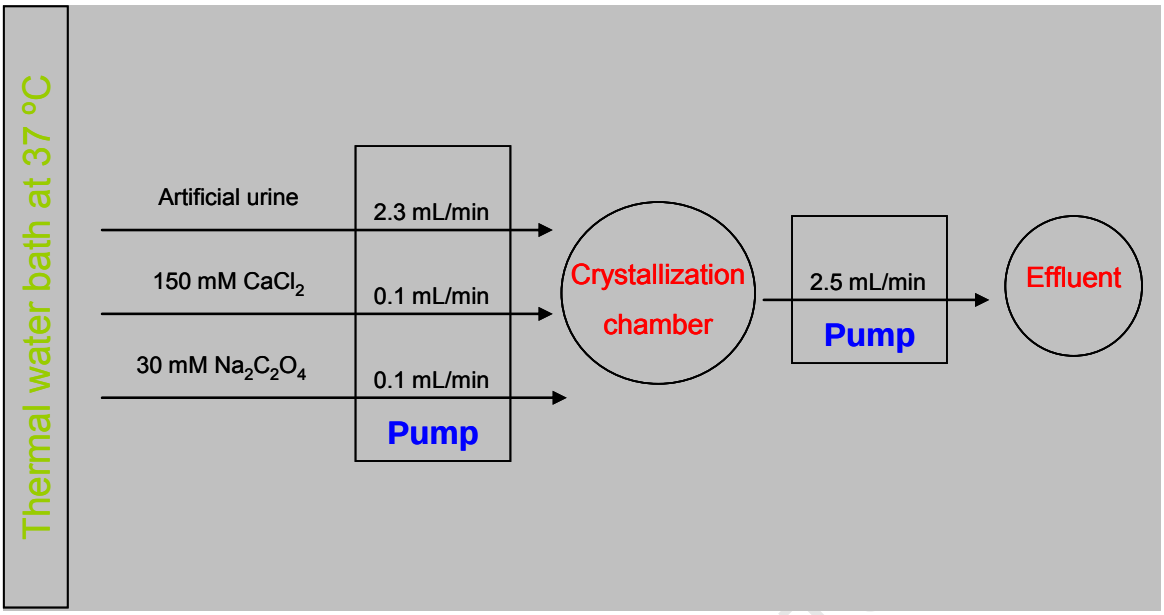


Figure 2.6: A schematic of the MSMPR system based on the design of the crystallizer used in this experiment

Calcium oxalate crystallization kinetics were measured after the crystallizer had been allowed to run 6-8 residence times (volume/flow rate) and had achieved steady state (dynamic equilibrium) (Rodgers and Garside 1981). Prior to steady state, the effluent from the MSMPR chamber was diverted to a waste tank. Two assumptions are made in this system namely that all crystals grow at the same rate and that aggregation occurs at a negligible rate. At steady state, CaOx crystals were taken from the product stream and the **number of particles** and **particle size distribution** were measured using a Coulter Multisizer 3 (*Coulter Electronics Ltd., England*).

At steady state, the crystal size distribution in the MSMPR crystallizer can be represented by the equation (Rodgers and Garside 1981):

$$n = B_0 \tau \, e^{(-L/G\tau)} \qquad (1)$$

Where

n = population density at steady state,

L = crystal size (μm),

B_0 = nucleation rate (number of particles.min^{-1}.mL^{-1}),

G = growth rate (μm.min^{-1}),

τ = mean residence time (minute)

By taking the natural logarithm on both sides, equation **1** becomes:

$$\text{Ln}(n) = \text{Ln}(B_0\tau) - L/(G\tau) \qquad (2)$$

The residence time (τ) which is the average time that crystals remain in the crystallizer, can be represented as:

$$\tau = (V/Q)$$

where V = volume of the chamber and Q = total flow rate.

By rearranging equation ***2***: Ln (n) = – L/Gτ + Ln (B_0τ), it can be represented in the form: y = mx + c. Therefore plotting the graph of Ln n (the number of crystals) vs. L (the crystal size) can facilitate the calculation of the growth rate (**G**) and nucleation rate (**B_0**). The growth rate (**G**) can be derived from the slope and nucleation rate (**B_0**) from the y-intercept. The y-intercept = Ln (B_0τ), therefore **B_0** = anti Ln (τ). The slope = -1/ Gτ, therefore **G** = -1/slope τ. An example of a graph of Ln n vs. L is shown in Figure 2.7.

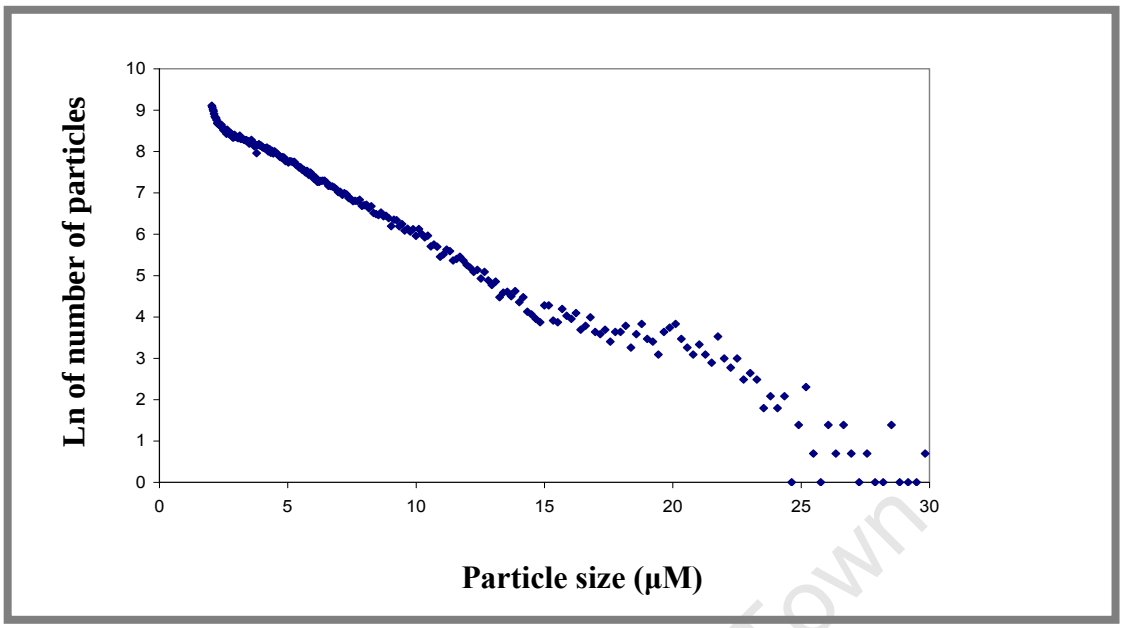


Figure 2.7: A typical plot of Ln of number of particles vs. particle size for the determination of nucleation (B_0) and growth (G) rates

Using the growth and nucleation rates, it is possible to calculate suspension density ($\mathbf{M_\tau}$, in $mg.L^{-1}$) according to the following equation (Kavanagh 1992):

$$M_\tau = \pi \rho B_0 G^3 \tau^4 x 10^{-6}$$

where ρ = crystal density ($g.cm^{-3}$)

Studying crystallization using the MSMPR system has advantages over other methods of measurements, as nucleation and growth rates are measured at the same time but independently of each other. It models the renal environment better than other crystallization techniques because it reaches steady state with a constant supersaturation and operates with a continuous flow. This allows individual crystals to transit the system quickly which is similar to the kidney (Kavanagh 1992).

2.10 Bonn risk index (BRI)

The BRI is an experimentally determined ratio of the concentration of free ionized calcium (mmol/L) to the concentration of ammonium oxalate (mol/100 mL) required to induce spontaneous crystallization of CaOx in the urine under study. It can be used as a diagnostic marker of the risk of stone formation (Laube *et al.* 2004). The BRI ratio is as follows:

$$\frac{[Ca^{2+}]}{[Ox^{2-}]} = \frac{\text{free ionized calcium (mmol/L)}}{\text{concentration of ammonium oxalate (mol/100 mL)}}$$

Urine samples with a BRI value < 1 are not at risk for kidney stone formation whereas BRI values > 1 indicate risk (Laube *et al.* 2004).

The initial concentration of free calcium in urine was determined by an ion selective electrode (*Photometer, Metrohm, Swiss*). Crystallization propensity was monitored in a 200 mL aliquot of urine by the step-wise addition of 0.4 M ammonium oxalate (0.5 mL per step and 1.5 ml/min). During the experiment, urine samples were maintained at 37 ^{o}C and continuously agitated. The onset of crystallization was detected *in situ* by a laser-probe crystal system analyzer (*Basic Titrino, Metrohm, Swiss*). This device determines the number of suspended particles *in vitro* and simultaneously estimates particle size in the detection range of 0.5-250 µm. The onset of crystallization is accompanied by a dramatic change in particle-size distribution, and therefore can be easily determined (Laube *et al.* 2000, Laube *et al.* 2004).

2.11 Urinary relative supersaturations

The urinary relative supersaturations (RS) of CaOx, brushite and uric acid were calculated using a modified version of EQUIL 2 by J. Asplin (*Litholink, Chicago, USA*) (Werness *et al.* 1985), a speciation programme which computes the equilibrium concentrations of complexes of primary cations and anions present in urine. Data from urine composition analysis were statistically analyzed by *Instat* and the results were reported statistically significant if $p \leq 0.05$. Average values were calculated and are reported with their standard error (SE).

2.12 Tiselius risk index (TRI)

The TRI is a ratio that indicates the biochemical risk of CaOx formation (Tiselius 1982). It is a mathematical expression given by:

$$\frac{(Ca/Cr)^{0.71} \times (Ox/Cr)}{(Mg/Cr)^{0.14} \times (Cit/Cr)^{0.10}}$$

where Ca, Ox, Mg and Cit are the urinary concentrations (mmol/24 hour) of calcium, oxalate, magnesium and citrate respectively. Cr is the urinary concentration (mol/24 hour) of creatinine. Typical values for normal urine lie in the range of 366 ± 14 while stone-formers' urine lie in the range 527 ± 17 (Tiselius 1982). However, this index is used more appropriately on a relative basis rather than by considering absolute values.

The effect of *Folium pyrrosiae, Desmodium styracifolium, Hylocerus trigonus, Phyllanthus niruri, Orthosiphon stamineus* and Cystone® on calcium oxalate crystallization in synthetic urine and real urine from black and white South African males

3.1 Introduction

In vitro CaOx crystallization experiments are of fundamental importance to urolithiasis research as the assessment of crystallization propensity in urine is considered an evaluation of risk (Hess *et al.* 2001). Different methods (either quantitative or qualitative) have been developed to imitate components of the renal system or aspects of the crystallization processes such as nucleation, aggregation and growth (Kavanagh 2006). Successful experiments are designed by matching the objective to the procedure (Hess *et al.* 1996). Experiments vary and are limited to the degree to which they mimic physiological conditions e.g. simple experiments performed in inorganic aqueous media to experiments carried out in real human urine which model features of urine flow dynamics (Kavanagh 1992, Hess *et al.* 2001).

As stated previously, crystallization is the process which drives the phase change from solution to solid. This process is governed by supersaturation i.e. a measure of the chemical potential between the 2 phases which directs all aspects of crystallization (Finlayson 1978, Hess and Kok 1996). As a reaction approaches equilibrium, the supersaturation decreases which impacts kinetic behaviour (Kavanagh 2006). Stone formation has been described as a result of an imbalance between 2 opposing influences i.e. urinary supersaturation and urinary inhibitors (Robertson *et al.* 1978a).

Human urine is a complex medium containing ions and macromolecules that can bind or complex with calcium and oxalate thereby modulating crystallization (Kok 1997, Hess *et al.* 2001). Therefore urinary saturation not only depends on calcium and oxalate but also on the presence of ions such as citrate and magnesium, as well as macromolecules such as glycoaminoglycans (Hess *et al.* 2001, Khan 2006).

This chapter describes *in vitro* studies of the plant extracts on CaOx crystallization.

3.2 Materials and methods

3.2.1 Preparation of plant extract stock solutions

Separate stock solutions (50 mL) of each herb were prepared by dissolving the dried powdered material in distilled water at 20 times the recommended dosage concentrations and used to dose urines at 5% (v/v) in order to achieve concentrations used in other studies or as recommended by the manufacturer. *Folium pyrrosiae* and *Desmodium styracifolium* were prepared at a concentration of 1.5 g/25 mL (Gohel *et al.* 2006). *Hylocerus trigonus* was prepared at a concentration of 20 g/25 mL (directions from supplier). *Phyllanthus niruri* was prepared at a concentration of 3.75 g/25 mL (Freitas *et al.* 2002) and *Orthosiphon stamineus* at a concentration of 2.5 g/25 mL (Arafat *et al.* 2008). Cystone® was prepared by dissolving 2 tablets in 25 mL distilled water (directions from supplier).

Only water extracts of the plant material were investigated. Experiments were performed in aqueous media. As such, organic extracts would have been insoluble. The plant material was weighed into conical flasks into which distilled water was added. Gentle heating with stirring (*Labcon, Johannesburg*) for 30 minutes was applied to facilitate decoction/dissolution. All stock solutions were microfiltered (0.22 μm) to remove insoluble material and stored in the refrigerator (7 ˚C) for up to 4 days.

3.2.2 Study design

Synthetic urine study

SU for this study was prepared as described in chapter 2.2. Experiments were carried out in SU alone, which served as a control, and SU dosed with the plant stock solution (5% (v/v)). The CaOx MSL and PSD of each urine were measured and crystals were examined by SEM.

Crystallization and inhibition kinetics were monitored by determining rates of nucleation, aggregation and growth of CaOx crystals are described in chapter 2. Data were statistically analyzed and p-values < 0.05 were regarded as significant.

Real urine study

Twenty-four hour urine samples were collected in plastic bottles from participants; exclusion criteria and details on how urine was processed are provided in chapter 2.3.

Investigation of the inhibitory properties of *Folium pyrrosiae* and *Desmodium styracifolium* on CaOx crystallization were performed using individual urine samples from black (n=7) and white (n=7) subjects. Crystallization parameters were measured in each urine sample and average values were determined.

Pooled urines from healthy black and white subjects were used to investigate the inhibitory properties of *Phyllanthus niruri*, *Orthosiphon stamineus* and Cystone® on CaOx crystallization. This change in study design, i.e. individual to pooled urines, was implemented to eliminate intra-racial urinary differences (such as urine chemistry and crystallization parameters) which were detected when analyzing individual urine samples. Each pooled urine per race group was constituted up by taking equal volumes (200 mL) of 24 hour urine sample from 5 subjects. Two urine pools were constituted for each race group whereby the same 5 subjects of each race group provided 24 hour urine samples on 2 occasions (*black pooled urines*: **BUP1** and **BUP2**; *white pooled urines*: **WUP1** and **WUP2**). Crystallization experiments were carried out on all 4 pooled urines. Data are reported separately. *Hylocerus trigonus* was excluded from the real urine studies due to solubility difficulties with the plant material at the required concentrations.

Experiments were carried out in urine alone, which served as a control, and urine dosed with the plant stock solution (5% (v/v)). Due to kinetic experiments being performed on different days the control values differed, therefore relative comparisons were made, meaning that an experimental value was compared with the control measured on that particular day. Raw data and plots of all experiments are presented in Appendix 1.

The CaOx MSL and PSD of each urine were measured and crystals were examined by SEM. Crystallization was monitored by determining rates and inhibition of nucleation, aggregation and growth of CaOx crystals. Crystal deposition rate and BRI were measured in urine samples. These techniques have been described in detail in chapter 2.

Experiments were repeated a minimum of three times; average values and standard error (SE) are reported. Error bars have been omitted from graphs for the sake of clarity. Data were statistically analyzed by *Graphpad Instat* and $p<0.05$ were regarded as significant. The following symbols were used to denote $p<0.05$:

* when comparing control (urine) versus experimental (urine dosed with plant extract) and

‡ ǂ when comparing black and white control urines

3.3 Results

3.3.1 Results of crystallization studies in synthetic urine

CaOx MSL

The mean MSL of undosed SU and SU dosed with *Folium pyrrosiae*, *Desmodium styracifolium*, *Hylocerus trigonus*, *Phyllanthus niruri*, *Orthosiphon stamineus* and Cystone® are presented in Table 3.1. No statistically significant changes to the MSL were detected in the presence of the plant extracts (PE).

Table 3.1: MSL (SE) of CaOx in SU in the presence and absence of plant extracts

	MSL of CaOx (µmol/l)	
	SU	***SU + PE***
Folium pyrrosiae	105 (0)	120 (0)
Desmodium styracifolium		120 (0)
Hylocerus trigonus	98 (2.58)	125 (0)
Phyllanthus niruri	75 (0)	75 (0)
Orthosiphon stamineus		105 (0)
Cystone®		90 (0)

CaOx PSD

The mean particle size of CaOx in undosed SU and SU dosed with plant extracts are presented in Table 3.2. *Desmodium styracifolium*, *Orthosiphon stamineus* and Cystone® caused a decrease ($p<0.05$) in average particle size.

Table 3.2: Average particle size (SE) of CaOx crystals in SU in the presence and absence of plant extracts

	Particle size mode (µm)	
	SU	***SU + PE***
Folium pyrrosiae	15.7 (0.53)	12.7 (0.70)
Desmodium styracifolium		12.3 (0.24) *
Hylocerus trigonus		18.5 (1.03)
Phyllanthus niruri	11.5 (0.38)	11.6 (0.67)
Orthosiphon stamineus		9.20 (0.60) *
Cystone®		7.95 (0.22) *

* *$p<0.05$*

SEM

Scanning electron micrographs were captured of CaOx crystals deposited in SU in the presence and absence of all plant extracts. Only those with apparent differences to undosed SU are presented in Figure 3.1. The total surface-area of each stub was examined and micrographs were recorded of typical deposits with respect to crystal size and morphology.

In SU alone (Figure 3.1a), mostly COM crystals are observed and some aggregates. In SU dosed with *Folium pyrrosiae*, larger crystals are observed than in undosed SU. Aggregates and smaller COM crystals are precipitated in the presence of *Folium pyrrosiae* (Figure 3.1b) and *Desmodium styracifolium* (Figure 3.1c). In the presence of *Hylocerus trigonus* (Figure 3.1d), an increase in nucleation is depicted by the large number of COM crystals observed and a decrease in crystal size was also noted. The most noteworthy change was induced by *Phyllanthus niruri* where mostly COD crystals precipitated in its presence (Figure 3.1e). No characteristic changes were detected in the presence of *Orthosiphon stamineus* and Cystone®.

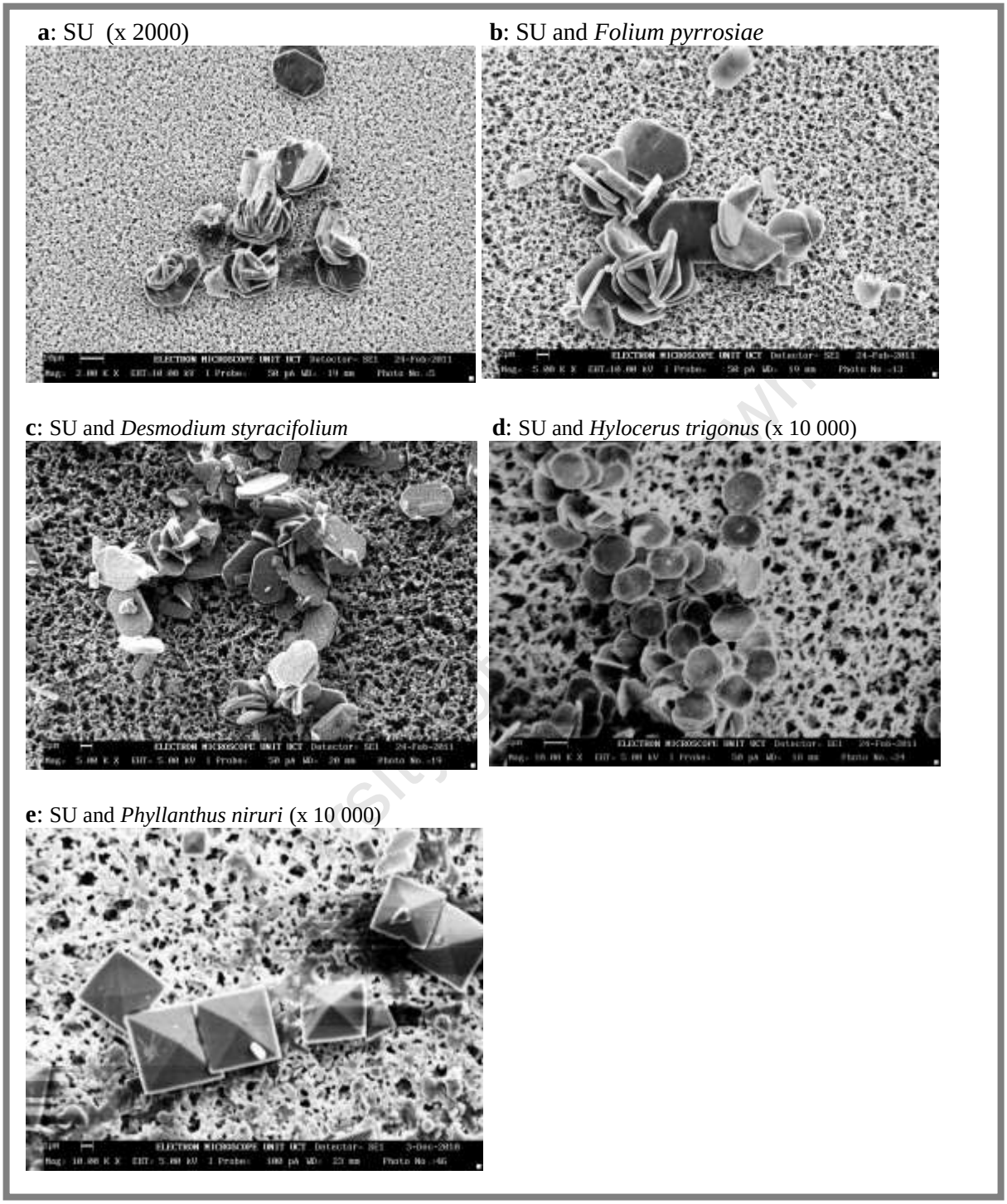


Figure 3.1: Electron micrographs of CaOx crystals deposited in SU alone and SU dosed with *Folium pyrrosiae*, *Desmodium styracifolium*, *Hylocerus trigonus* and *Phyllanthus niruri*. Micrographs are at 5000 x magnification unless otherwise stated

Simultaneous measurement of CaOx crystal nucleation and aggregation kinetics

The percentage inhibitions of CaOx crystal nucleation and aggregation in the presence and absence of plant extracts are presented in Table 3.3. A negative percentage indicates promotion. The general trend noted is that SU inhibits nucleation and promotes aggregation. Of all the plant extracts, only Cystone® induced a statistically significant increase in the promotion of nucleation ($p<0.05$). With respect to crystal aggregation, the presence of *Hylocerus trigonus* and *Orthosiphon stamineus* caused a decrease in the inhibition this mechanism ($p<0.05$).

Table 3.3: Percentage inhibitions (SE) of CaOx nucleation and aggregation in SU in the presence and absence of plant extracts

	% Inhibition of NUCLEATION		% Inhibition of AGGREGATION	
	SU	***SU + PE***	***SU***	***SU + PE***
Folium pyrrosiae	31.8 (21.2)	40.8 (18.5)	-37.6 (9.82)	-37.3 (14.6)
Desmodium styracifolium		38.9 (19.1)		-23.7 (7.17)
Hylocerus trigonus	22.9 (15.2)	22.1 (14.0)	-34.8 (4.38)	-12.9 (5.8) *
Phyllanthus niruri	23.9 (39.5)	41.3 (30.4)	-32.3 (2.67)	3.01 (1.94)
Orthosiphon stamineus		19.3 (29.4)		23.7 (36.6) *
Cystone®		-35.5 (46.1) *		-71.0 (3.29)

* *p<0.05*

CaOx crystal growth kinetics

The percentage inhibition of CaOx crystal growth in the presence and absence of plant extracts are presented in Table 3.4; a negative percentage indicates promotion. *Desmodium styracifolium*, *Phyllanthus niruri*, *Orthosiphon stamineus* and Cystone® induced statistically significant increases in the inhibition of growth of CaOx crystals in SU ($p<0.05$).

Table 3.4: Percentage inhibitions of CaOx growth in SU in the presence and absence of plant extracts

	% Inhibition of GROWTH	
	SU	***SU + PE***
Folium pyrrosiae	35.1 (2.7)	31.8 (4.2)
Desmodium styracifolium		56.1 (3.8) *
Hylocerus trigonus	43.4 (12.0)	46.1 (12.6)
Phyllanthus niruri	-56.9 (67.2)	23.5 (33.9) *
Orthosiphon stamineus		15.2 (36.6) *
Cystone®		2.22 (42.2) *

* *p<0.05*

Measurement of CaOx crystal nucleation and growth rates by the MSMPR crystallizer

Nucleation and growth rates of CaOx crystals which were measured by means of an MSMPR crystallizer are presented in Figures 3.2 and 3.3 respectively. *Phyllanthus niruri*, *Orthosiphon stamineus* and Cystone® caused statistically significant increases in the rate of nucleation ($p<0.05$). All plant extracts induced a statistically significant decrease in crystal growth ($p<0.05$).

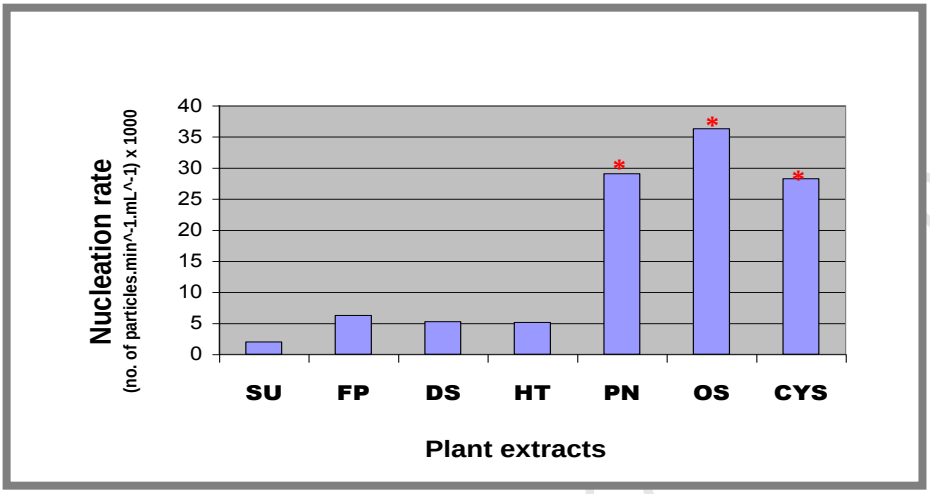


Figure 3.2: Rate of CaOx crystal nucleation in SU in the presence and absence of the plant extracts (* *p<0.05*)

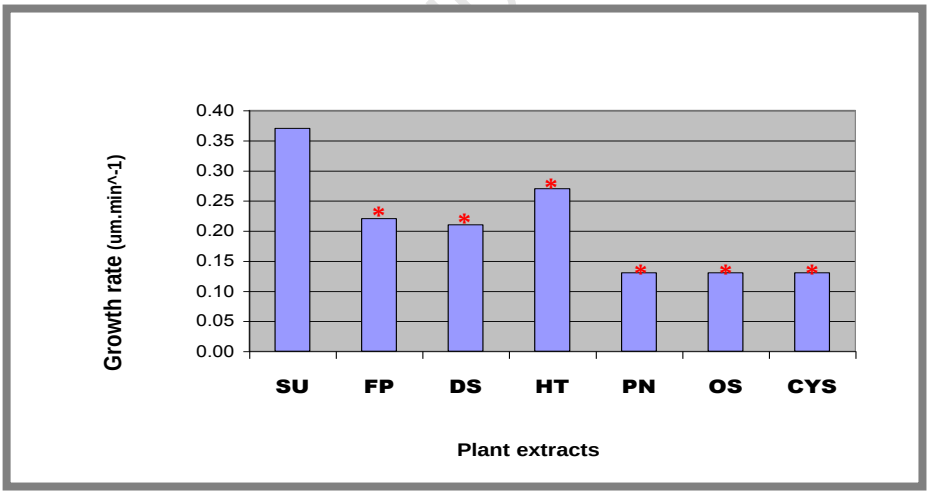


Figure 3.3: Rate of CaOx growth in SU in the presence and absence of the plant extracts (* *p<0.05*)

3.2.2 Results of crystallization studies in real urine

Data for the inhibitory studies of *Folium pyrrosiae* and *Desmodium styracifolium* on CaOx crystallization are reported as the mean values (n=7) obtained from the analysis of 24 hour urine obtained from healthy black (**BU**) and white (**WU**) subjects. Inhibitory activity of *Phyllanthus niruri*, *Orthosiphon stamineus* and Cystone® on CaOx crystallization was determined on pooled urines. As mentioned previously, two urine pools were constituted for each race group and data are reported separately. (*Black pooled urines*: **BUP1** and **BUP2**; *White pooled urines*: **WUP1** and **WUP2**).

CaOx MSL

The mean CaOx MSL of BU and WU are reported in Table 3.5. The presence of the plant extracts caused no significant changes. However, the MSL of black urine is significantly higher ($p<0.05$) than that of whites in pooled and individually measured samples.

Table 3.5: MSL (SE) of CaOx in BU and WU in the presence and absence of plant extracts

	MSL of CaOx (µmol/l)			
	BU	***BU + PE***	***WU***	***WU + PE***
Folium pyrrosiae	105 (6.6) ‡	107 (6.9)	71 (12.9) ‡	64 (7.8)
Desmodium styracifolium		107 (8.3)		62 (6.9)

	MSL of CaOx (µmol/l)							
	BUP1	***BUP1 + PE***	***BUP2***	***BUP2 + PE***	***WUP1***	***WUP1 + PE***	***WUP2***	***WUP2 + PE***
Phyllanthus niruri	120 (0) ‡	115 (0)	75 (0) ŧ	75 (0)	45 (0) ‡ŧ	45 (0)	45 (0) ‡ŧ	60 (0)
Orthosiphon stamineus		105 (0)		60 (0)		45 (0)		45 (0)
Cystone®		120 (0)		60 (0)		45 (0)		45 (0)

*$p<0.05$: * when comparing control (urine) versus experimental (urine dosed with plant extract)*
‡ ŧ when comparing black and white control urines

CaOx PSD

The average particle size of CaOx crystals in BU and WU, in the presence and absence of *Folium pyrrosiae, Desmodium styracifolium, Hylocerus trigonus, Phyllanthus niruri, Orthosiphon stamineus* and Cystone® are reported in Table 3.6. No significant changes in particle size were induced by the plant extracts. There were also no significant differences in the average particle size of CaOx crystals precipitated from the urine of the two race groups i.e. in undosed BU and WU.

Table 3.6: Average particle size (SE) of CaOx in BU and WU in the presence and absence of plant extracts

	Particle size mode (µm)			
	BU	***BUP1 + PE***	***WU***	***WUP1 + PE***
Folium pyrrosiae	10.9 (1.6)	10.1 (0.6)	11.6 (0.5)	11.1 (0.5)
Desmodium styracifolium		10.6 (1.3)		11.7 (0.7)
	Particle size mode (µm)			
	BUP2	***BUP2 + PE***	***WUP2***	***WUP2 + PE***
Phyllanthus niruri	9.3 (1.1)	9.7 (0.8)	10.7 (1.2)	7.4 (0.6)
Orthosiphon stamineus		7.8 (0.8)		7.7 (0.5)
Cystone®		9.4 (0.1)		7.5 (0.4)

SEM

Scanning electron micrographs were recorded of CaOx crystals deposited in SU in the presence and absence of all plant extracts. Only those with apparent differences to undosed SU are presented. The total surface-area of each stub was examined and micrographs were recorded of typical deposits with respect to crystal size and morphology

Figure 3.4 shows the scanning electron micrographs of CaOx crystals deposited in BU and BU dosed with *Phyllanthus niruri*, *Orthosiphon stamineus* and Cystone®. A large number of crystals and mostly COM precipitate in the BU control (Figure 3.4a). In the presence of these plant extracts, a large number of crystals of seemingly smaller size are observed. Upon the addition of *Phyllanthus niruri* more COD crystals are detected (Figure 3.4b).

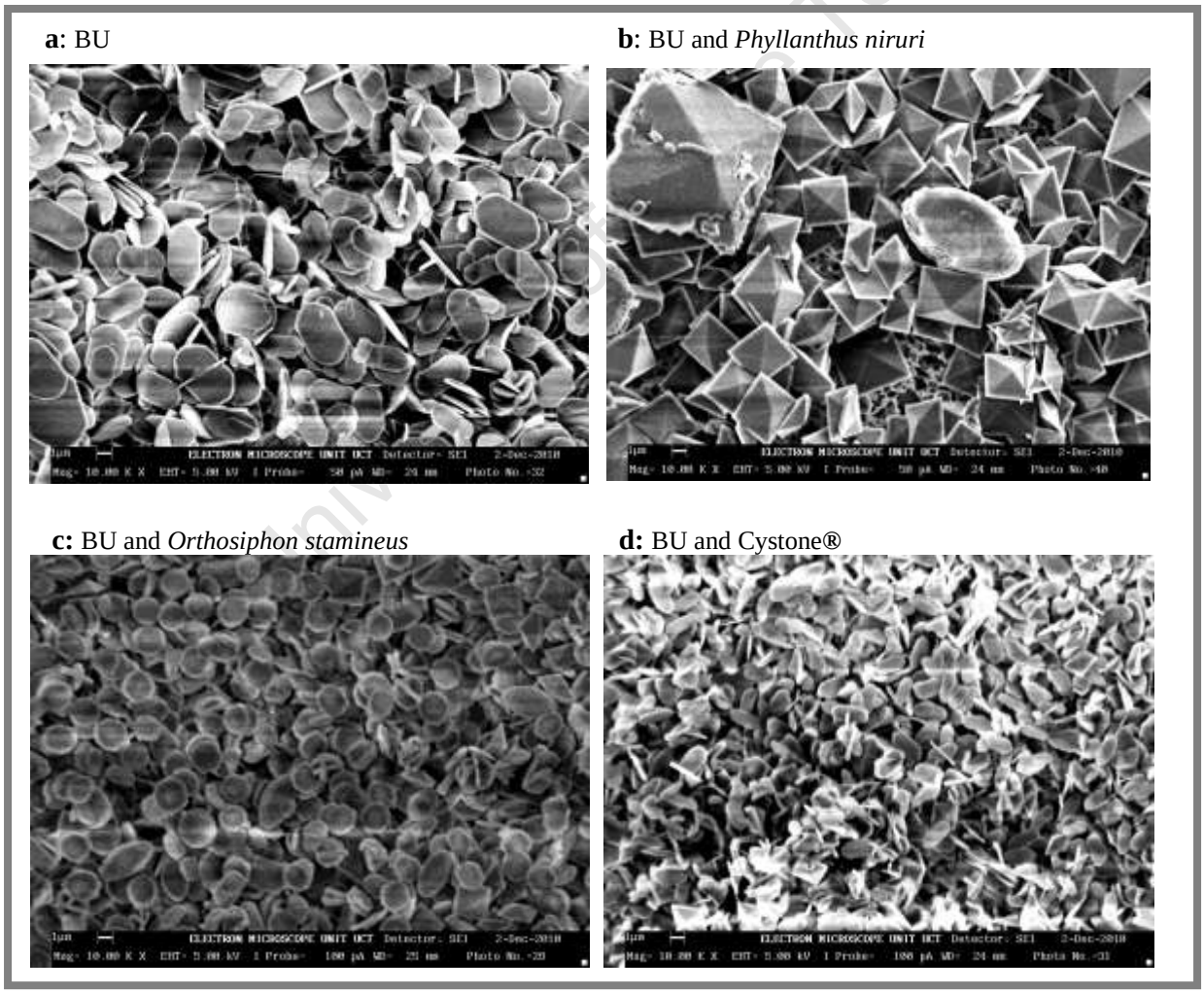


Figure 3.4: Electron micrographs of CaOx crystals deposited in BU. Micrographs are at 10 000X magnification

Figure 3.5a and b shows the scanning electron micrographs of CaOx crystals deposited in WU. Fewer but seemingly larger crystals precipitate in WU compared to BU and a mixture of COM and COD are observed. In the presence of *Orthosiphon stamineus* (Figure 3.5c) and Cystone (Figure 3.5d), only COD crystals can be viewed which are smaller than those in undosed WU.

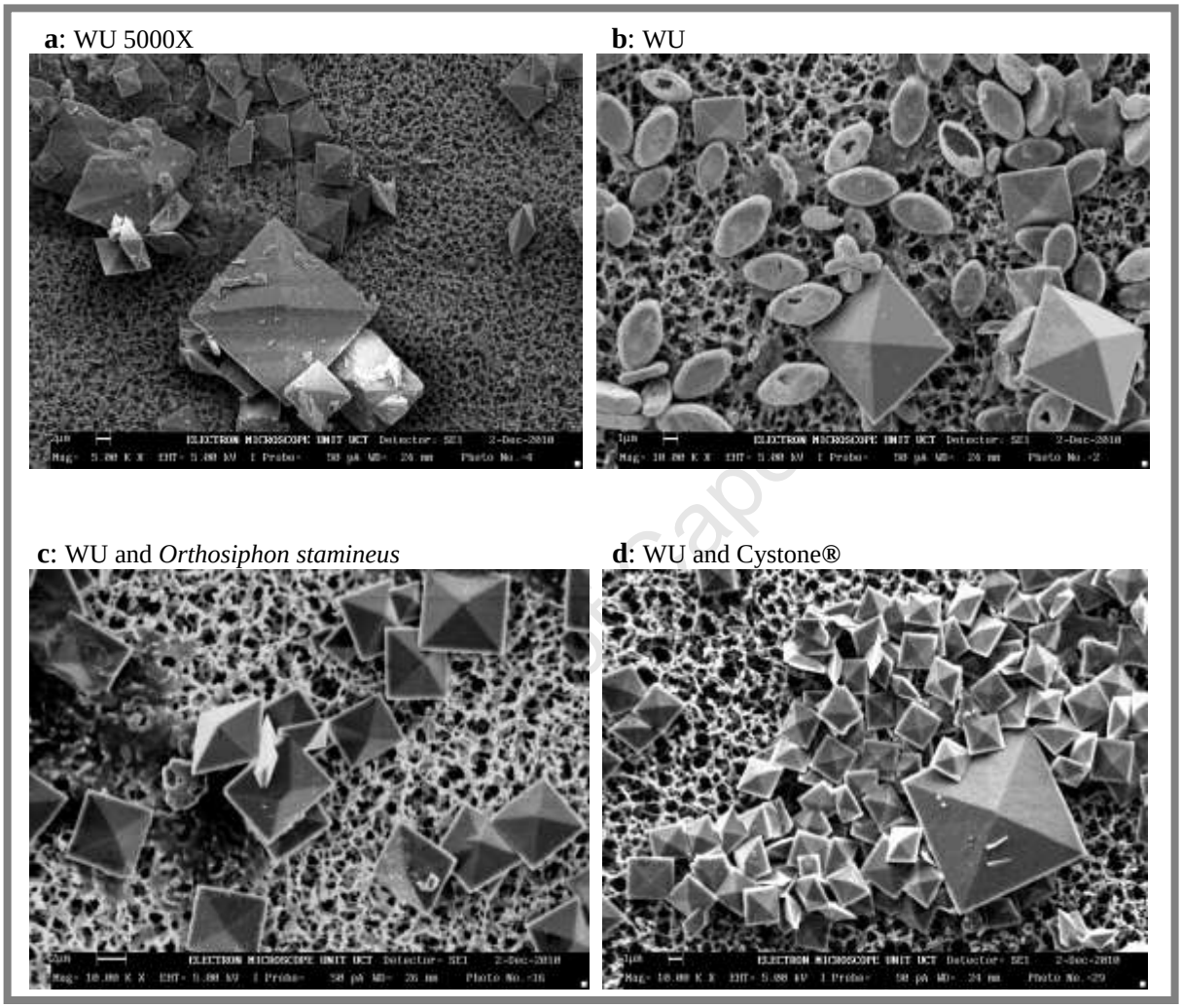


Figure 3.5: Electron micrographs of CaOx crystals deposited in WU. Micrographs are at 10 000X magnification unless otherwise stated

Simultaneous measurement of CaOx crystal nucleation and aggregation kinetics

The inhibition of CaOx crystal nucleation and aggregation has been calculated and presented in Tables 3.7a and b respectively. Results of the pooled urine studies are reported separately.

Folium pyrrosiae and *Desmodium styracifolium* showed no effect on crystal nucleation and aggregation in either race group. In BU, the only significant change detected in BUP2 where *Orthosiphon stamineus* induced an increase in the inhibition of aggregation ($p<0.05$), Table 3.7b. In WU, no significant changes were detected in WUP1 however in WUP2 *Orthosiphon stamineus* caused a decrease in the promotion of nucleation ($p<0.05$) and Cystone® caused a decrease in inhibition of aggregation ($p<0.05$).

Comparison of BU and WU controls shows that inhibition of crystal aggregation is greater WU ($p<0.05$). This result was consistent in both pools.

Table 3.7a: Percentage inhibitions (SE) of CaOx nucleation in BU and WU in presence and absence of plant extract

	% Inhibition of NUCLEATION			
	BU	***BU + PE***	***WU***	***WU + PE***
Folium pyrrosiae	-66.6 (40.9)	-45.5 (37.3)	-2.01 (43.7)	13.7 (44.9)
Desmodium styracifolium		-44.8 (39.9)		21.1 (43.5)

	% Inhibition of NUCLEATION							
	BUP1	***BUP1 + PE***	***BUP2***	***BUP2 + PE***	***WUP1***	***WUP1 + PE***	***WUP2***	***WUP2 + PE***
Phyllanthus niruri	-18.9 (14.3)	-80.2 (31.4)	-9.6 (34.4)	18.1 (12.1)	-39.9 (3.3)	-41.1 (5.5)	-32.4 (3.3)	-7.27 (2.2)
Orthosiphon stamineus		-38.3 (15.7)		4.92 (13.7)		-57.5 (6.8)		-17.5 (4.3) *
Cystone®		-25.1 (8.9)		-6.98 (16.9)		-35.9 (3.5)		-7.22 (0.05)

*$p<0.05$: * when comparing control (urine) versus experimental (urine dosed with plant extract)*
‡ ǂ when comparing black and white control urines

Table 3.7b: Percentage inhibitions (SE) of CaOx aggregation in BU and WU in presence and absence of plant extract

	% Inhibition of AGGREGATION			
	BU	***BU + PE***	***WU***	***WU + PE***
Folium pyrrosiae	-4.71 (65.4)	-0.79 (46.3)	39.0 (33.5)	50.2 (40.2)
Desmodium styracifolium		-31.8 (44.4)		9.98 (37.8)

	% Inhibition of AGGREGATION							
	BUP1	***BUP1 + PE***	***BUP2***	***BUP2 + PE***	***WUP1***	***WUP1 + PE***	***WUP2***	***WUP2 + PE***
Phyllanthus niruri	-16.8 (0.63) ‡	-61.6 (1.3)	17.3 (0.9) ǂ	50.0 (0.4)	41.2 (28.5) ‡ǂ	40.9 (0.4)	41.8 (0.4) ‡ǂ	41.7 (0.6)
Orthosiphon stamineus		-56.7 (0.8)		65.9 (0.5) *		14.9 (0.3)		24.3 (0.2)
Cystone®		-60.1 (0.9)		21.7 (0.7)		18.6 (0.4)		14.5 (0.3) *

*p<0.05: * when comparing control (urine) versus experimental (urine dosed with plant extract)*
‡ ǂ when comparing black and white control urines

[^{14}C]-oxalate deposition experiments

Precipitated [^{14}C]-oxalate as a function of time in BU and WU are presented in Figure 3.6. All samples [BU (n=3) & WU (n=3); BUP2 and WUP2] were determined in triplicate and at the end of each experiment, the precipitated [^{14}C]-oxalate was determined in duplicate using a scintillation counter. Hence each data point presented in the respective graphs is the average of 6 measurements (except for BUP2 + PN where each data point is an average of 2 measurements as the other data points were omitted due to experimental error).

The rate of precipitation is represented by the gradient of the linear portion of each curve. In undosed BU (Figure 3.6a and b) there is an increase in the precipitation of CaOx overall, however initially there is an increasing rate followed by a plateau from 60 – 90 minutes, and then a slight increase in the rate again from 90 – 120 minutes. In BU, statistical comparisons of the slopes between 0 – 60 minutes showed that the addition of *Desmodium styracifolium* and *Orthosiphon stamineus* significantly decreased the rate of precipitated CaOx ($p<0.05$). None of the other plant extracts demonstrated any significant effect over the course of the experiment.

In undosed WU (Figure 3.6a and d), there is a continuous increase in the precipitation of CaOx, and the rate gradually decreased over the 2 hour period as depicted by the shape of the graph i.e. decreasing concavity. In WU, the presence of *Phyllanthus niruri* induced an increase ($p<0.05$) in the rate of precipitation between 0 – 30 minutes and the presence Cystone® induced an increase ($p<0.05$) in the rate of precipitation between 0 – 60 minutes. No significant effect on CaOx precipitation was exhibited by any of the other plant extracts in WU.

Comparison of the BU and WU controls in Figure 3.6 shows that at the end of the experimental period (120 minutes), CaOx precipitation was greater in WU i.e. a greater particle volume.

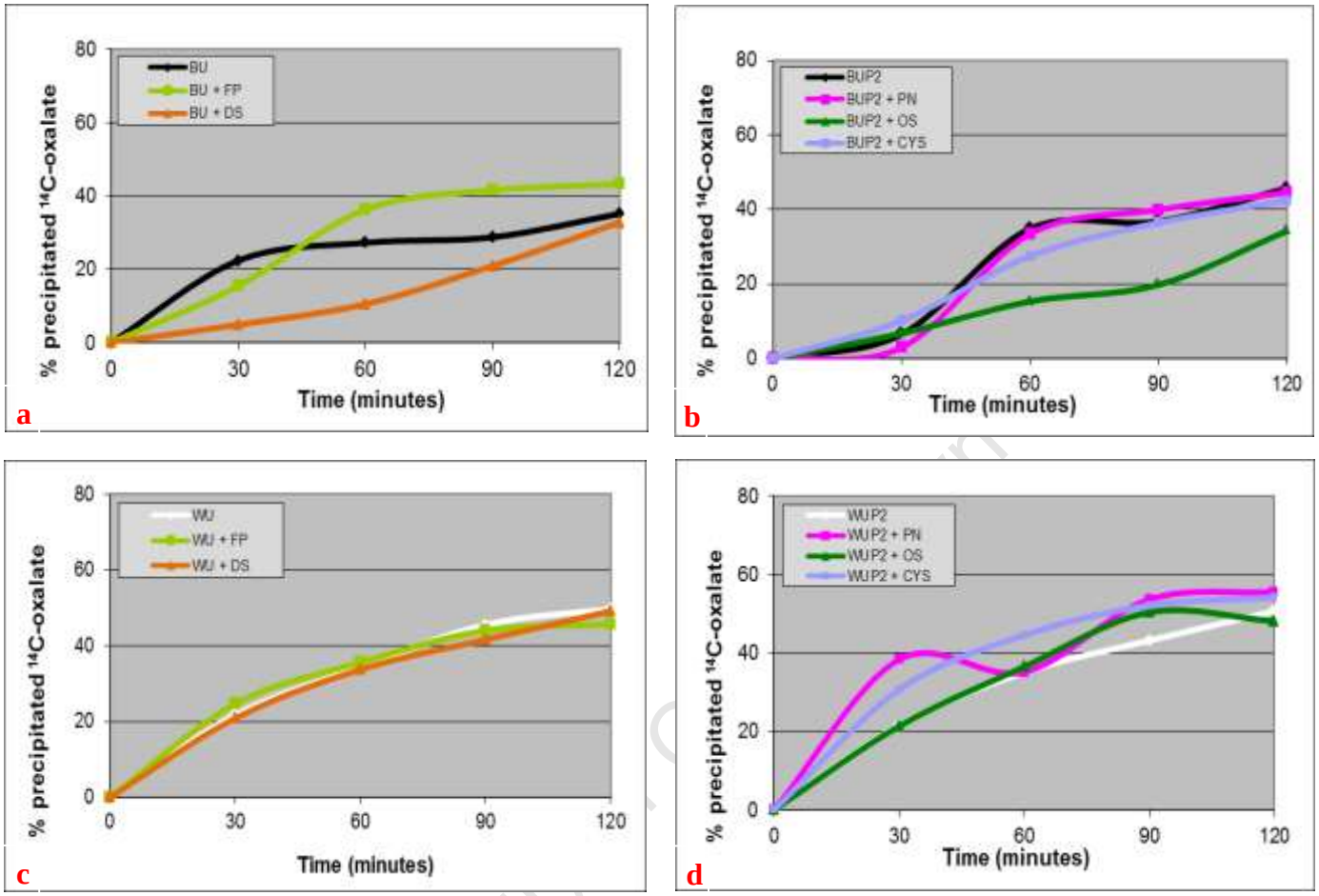


Figure 3.6a-d: Rate of [^{14}C]-oxalate deposition in individual and pooled BU and WU in the presence and absence of plant extracts

BRI

Mean BRI values for both race groups are given in Table 3.8. The addition of *Folium pyrrosiae* and *Desmodium styracifolium* to BU and WU did not cause any significant change in the BRI. It is noted that BRI of urine from the black group is lower than that of the white group and in both race groups the BRI is less than 1.

Table 3.8: Mean BRI values (SE) in the presence and absence of plant extracts

	BRI			
	BU	***BU + PE***	***WU***	***WU + PE***
Folium pyrrosiae	0.553 (0.086)	0.450 (0.050)	0.787 (0.273)	0.706 (0.239)
Desmodium styracifolium		0.514 (0.057)		0.798 (0.281)

3.2.3 *Summary of the effects of the plant extracts on* in vitro *CaOx crystallization*

Table 3.8: Summary of the significant effects ($p<0.05$) of *Folium pyrrosiae*, *Desmodium styracifolium*, *Hylocerus trigomus*, *Phyllanthus niruri*, *Orthosiphon stamineus* and Cystone® on *in vitro* CaOx crystallization

	SYNTHETIC URINE									
	PARTICLE SIZE	SEM		% INHIBITION			MSMPR: KINETICS OF			
		SIZE	NUMBER	NUC	AGG	GROWTH	NUC	GROWTH		
Folium pyrrosiae		↓						↓		
Desmodium styracifolium	↓	↓				↑		↓		
Hylocerus trigonus		↓	↑		↑			↓		
Phyllanthus niruri						↑	↑	↓		
Orthosiphon stamineus	↓				↑	↑	↑	↓		
Cystone ®	↓			↓		↑	↑	↓		

	BLACK URINE					WHITE URINE				
	SEM		% INHIBITION			SEM		% INHIBITION		
	SIZE	NUMBER	NUC	AGG		SIZE	NUMBER	NUC	AGG	
Folium pyrrosiae										
Desmodium styracifolium										
Phyllanthus niruri	↓	↑								
Orthosiphon stamineus	↓	↑		↑		↓		↑		
Cystone ®	↓	↑				↓				

↓ *denotes a decrease*
↑ *denotes an increase*

3.4 Discussion

Synthetic urine study

The value of MSL is a measure of crystallization propensity in a solution. None of the plant extracts had an effect on this property. However once crystallization was initiated, the plant extracts had an effect on the mechanisms of crystallization. These effects are summarized in Table 3.8.

Desmodium styracifolium, *Orthosiphon stamineus* and Cystone® caused a decrease in the average particle-size of CaOx crystals (revealed by the particle size-volume distribution), which can arise as a result of inhibition of crystal growth. Only Cystone® triggered a promotion of crystal nucleation whereas *Hylocerus trigonus* and *Orthosiphon stamineus* induced a decrease in crystal aggregation as revealed in the simultaneous nucleation and aggregation assay. An increase in nucleation is considered prophylactic as it rapidly reduces supersaturation when crystal size is simultaneously reduced (Kavanagh 1992).

Supplementary evidence, to the particle-size data presented above, of the inhibitory effects of *Desmodium styracifolium*, *Orthosiphon stamineus* and Cystone® on crystal growth was provided by the growth assay. This experiment also implicated *Phyllanthus niruri* as an inhibitor of crystal growth.

Results of kinetic studies performed in the MSMPR crystallizer revealed that *Phyllanthus niruri*, *Orthosiphon stamineus* and Cystone® induced an increase in nucleation rate and all the plant extracts under investigation instigated a decrease in crystal growth rate. Some of the plant extracts did not exhibit any effect on crystal nucleation and growth when investigated by spectroscopic assays but the MSMPR system is considered to be the most credible method of measuring *in vitro* crystallization (Hess *et al.* 2001). Therefore these are all highly favourable outcomes in the context of urolithiasis as each represents a reduced risk of stone formation.

It was observed in scanning electron micrographs that the presence of *Phyllanthus niruri* in SU favoured the crystallization of COD crystals exclusively. COD crystals are known to have

a lower affinity for adhesion to renal epithelial cells (Wesson *et al.* 1998) hence minimizing the risk of stone formation. In addition, the higher positive charge on COD results in greater repulsive forces between crystals and therefore favors disaggregation (Webber *et al.* 2003). Therefore, the formation of COD crystals in the present study is another highly favourable outcome.

Real urine study

Interesting trends have been noted upon comparison of urine collected from the two race groups. In both sets of pooled urines (BUP1, BUP2 and WUP1, WUP2), the CaOx MSL was found to be higher in black subjects indicating that crystallization is initiated less easily in this race group – a finding that is consistent with the rarity of stones in the black population. However, none of the plant extracts exhibited an effect on the MSL of urine from either race group. This is not uncommon as many other well-established inhibitors have also demonstrated no effect on this parameter e.g. citrate (Ryall *et al.* 1985), urinary glycosaminoglycans e.g. heparin sulphate (Suzuki and Ryall 1996) and proteins e.g. Tamm-Horsfall mucoprotein and prothrombin fragment 1 (Grover *et al.* 1994, Grover and Ryall 2002).

The only quantifiable significant effect induced by the plant extracts in white urine was found in WUP2 where *Orthosiphon stamineus* initiated an increase in the inhibition of nucleation (Table 3.6a). Two changes in crystallization parameters were demonstrated in black urine: *Orthosiphon stamineus* lead to increased inhibition of aggregation and Cystone® resulted in decreased inhibition of aggregation.

Another distinction between the urinary responses in black and white subjects is a lower inhibition of aggregation in the former group. This finding is counter-intuitive as a lower inhibition of aggregation is a risk factor and would make black subjects more susceptible to stone formation.

Crystal deposition experiments showed a larger particle volume in undosed WU (which is a reflection of crystal aggregation) compared to BU after a 120 minute incubation period. None of the plant extracts showed a significant effect on this property. However they affected the kinetics

of deposition as determined by the [^{14}C]-deposition experiment. *Desmodium styracifolium* and *Orthosiphon stamineus* decreased the rate of CaOx precipitation in BU while *Phyllanthus niruri* and Cystone® induced an increase in the rate of precipitation in WU. A decrease in the rate of deposition is favourable as it reduces the risk of stone formation.

A difference is the morphology of urinary CaOx crystals isolated from the two race groups was observed by SEM. A mixture of COM and COD is observed in WU whereas almost exclusively COM precipitates in BU. The precipitation of COD crystals in WU is likely due to white subjects having a higher urinary calcium concentration which as a result favours the formation of these crystals (Webber *et al.* 2002). Fewer but seemingly larger crystals precipitate in WU compared to BU which would suggest that the former inhibits nucleation and promotes crystal growth, indicating a higher risk of stone formation in that race group. Data from the particle-size experiment also showed that the average size of crystals in WU to be non-significantly greater than BU.

The addition of *Phyllanthus niruri* to BU favours the deposition of COD (as was the case in SU). The clinical significance of these crystals has been mentioned earlier in this discussion. Crystal sizes have decreased in the presence of *Orthosiphon stamineus* and Cystone®. Scanning electron micrographs of undosed WU showed predominance of COD however upon addition of *Orthosiphon stamineus* and Cystone® exclusively COD was viewed.

Although the mechanisms of crystallization i.e. nucleation, aggregation and growth are quantified independently in these *in vitro* studies, under physiological conditions these processes cannot be separated (Kavanagh 2006). However obtaining these data was necessary to provide a foundation upon which further experiments were planned.

The summarized results in Table 3.8 allow for comparison of the relative strengths of the plant extracts. Based on the number of significant inhibitory effects demonstrated, *Orthosiphon stamineus* and Cystone® are the greatest potential inhibitors of CaOx crystallization, followed by (in decreasing order of inhibitory action) *Hylocerus trigonus*, *Desmodium styracifolium*, *Phyllanthus niruri* and *Folium pyrrosiae*.

The *in vitro* inhibitory activity of *Folium pyrrosiae*, *Desmodium styracifolium* and *Phyllanthus niruri* in SU observed in this study is consistent with what has previously been reported (Hirayama *et al.* 1993, Freitas *et al.* 2002, Barros *et al.* 2003, Barros *et al.* 2006, Gohel *et al.* 2006). To date, data on the effects of *Hylocerus trigonus*, *Orthosiphon stamineus* and Cystone® on *in vitro* CaOx crystallization have not been published.

Fewer effects were observed in crystallization parameters when moving from synthetic to real urine suggesting caution when extrapolating these results to physiological conditions. However *Orthosiphon stamineus* and Cystone® again demonstrated the strongest inhibitory action in real urine. Different physico-chemical responses were induced by the plant extracts in urines from the two South African population groups (evident in Table 3.8) indicating that urine chemistries in the two groups elicit different reactions. This confirms what has been reported previously in the literature (mentioned in section 1.5).

However a promising result of the activity of the plant extracts, which was demonstrated in both real and synthetic urine, is their ability to favour the crystallization of COD crystals (over COM). These crystals are well-known for decreased binding affinity to renal epithelial cells. Therefore studies on crystal-cell binding, testing the effect of the plant extracts were warranted. Additionally, in view of the empirically observed differences in the crystallization processes in the urines from black and white subjects, the aforementioned study involving crystal binding affinities is also justified. These will be described in the next chapter.

The effect of traditional herbs: *Folium pyrrosiae, Desmodium styracifolium, Hylocerus trigonus, Phyllanthus niruri, Orthosiphon stamineus* and Cystone on calcium oxalate crystal binding to MDCK-I cells

4.1 Introduction

The study of intracellular crystals and crystal-cell binding *in vivo* is limited. Histological examination of renal tissue from stone patients is challenging as calcium deposits upset cellular architecture (Lieske *et al*. 1996a), therefore cultured renal epithelial cell lines have become a powerful tool in investigating the mechanisms of crystal-cell binding (Hackett *et al*. 1994, Lieske *et al*. 1994, Goswami *et al*. 1995, Verkoelen *et al*. 1995, Lee *et al*. 1996, Verkoelen *et al*. 1999, Rabinovich *et al*. 2006).

Cultured epithelial cells fall into three categories namely primary, secondary and continuous cell cultures. Primary cell cultures are derived directly from the tissue under investigation. Secondary or 'semi-continuous' cell cultures can grow and divide for a limited time in culture, whereas continuous or 'immortalized' cell cultures can grow indefinitely in culture (Price *et al*. 1994, Bigelow *et al*. 1998).

Previous studies have shown that immortalization of a cell line can induce changes in cell morphology and gene expression (Brezden and Rauth 1994, Verkoelen *et al*. 1997). There is also variation in COM crystal binding affinity for the different cell lines (Bigelow *et al*. 1998, Verkoelen *et al*. 1999). COM crystals show the greatest binding affinity for continuous Madin-Darby canine kidney (MDCK) type I cells. In addition, there is no significant change in the crystal-cell binding after their continuous culture for 9 days (Bigelow *et al*. 1998). The advantage of semi-continuous and continuous cell lines over primary cell lines is that they are convenient to work with and they can be stored frozen and additional experiments can be carried out later with the same material (Verkoelen *et al*. 1997). Therefore MDCK-I cells are considered suitable for investigating the mechanisms of crystal-cell binding (also referred to as attachment) and various studies have been reported (for example: Lee *et al*. 1996, Thamiselvan *et al*. 2000, Schepers *et al*. 2002, Kumar *et al*. 2003, Atmani *et al*. 2004, Rabinovich *et al*. 2006,).

As mentioned in Chapter 1, the pathogenesis of renal stones involves the combination of two processes, namely nucleation of crystals and their retention within the kidney which allows for further growth and/or aggregation to occur. Urine in the distal region of the nephron is supersaturated with calcium and oxalate ions and spontaneous nucleation of crystals follows (Finlayson *et al*. 1978a, Verkoelen *et al*. 1997, Kumar *et al*. 2003, Grover *et al*. 2008).

The 'free particle' and 'fixed particle' theories have been proposed to explain stone formation within the renal system. Although both theories are credible, this thesis supports the fixed particle mechanism (Finlayson *et al*. 1978a, Fleisch 1978, Hess and Kok 1996). Previously reported rates of urine flow and crystal growth indicate that it is not possible for a single crystal (*i.e.* 'free' particle) to grow large enough to obstruct the flow of urine through the nephron (Kok *et al*. 1986, Kok *et al*. 1990a, Kok and Khan 1994). Therefore crystals require an attachment site within the kidney (Finlayson *et al*. 1978a, Coe and Parks 1988, Kumar *et al*. 2003, Tiselius 2011). Studies have shown that COM crystals can bind irreversibly to the surface of renal epithelial cells and this is considered to be a critical process in the growth of renal calculi (Finlayson *et al*. 1978a, Lieske *et al*. 1992a, Lieske *et al.* 1992b, Ebisuno *et al*. 1995, Verkoelen *et al*. 1996, Verkoelen *et al*. 2000). The latter finding prompted a series of investigations into factors affecting the interaction between COM crystals and renal epithelial cells and the following has been reported.

The most prominent morphology of CaOx that occurs in human stones is COM. These crystals are highly membranolytic and irreversibly bind to renal epithelial cells (Lieske *et al*. 1999). Binding occurs very rapidly, is concentration dependent and is significantly greater than that of other calcium-containing crystals (Lieske *et al*. 1999, Lieske and Toback 2000, Grover *et al*. 2008). Furthermore it has been reported that following cell binding, CaOx crystals undergo internalization and subsequent dissolution (Lieske *et al*. 1999).

Once crystals attach to the surface of cells, some are taken up by the cells (Lieske and Toback 1993, Lieske *et al*. 1994, Verkoelen *et al*. 1995). The fate of the endocytosed crystals has not been established with certainty. It has been hypothesized that this could represent the initial stages of stone development (Verkoelen *et al*. 1997), whereas others believe that this could

represent a protective mechanism to remove potential growth sites from the cell surface (Lieske *et al*. 1992b, Lieske and Toback 1993). Ultrastructural studies of MCDK cells revealed that initially endocytosed crystals had disappeared within 72 hours and it has been suggested that crystals undergo dissolution intracellularly due to the presence of lysosomes (Goswami *et al*. 1995). In studies of hyperoxaluric patients, CaOx crystals were found in tubular lumen as well as in the renal interstitium which could be a result of translocation of luminal crystals to the interstitial space (Morgenroth *et al*. 1968, Saxon *et al*. 1974, Wharton *et al*. 1990, Lieske *et al*. 1992a, Tiselius 2011). However these processes of dissolution and translocation take extended periods to occur (hours up to days) (Lieske and Toback 1993, Lieske *et al*. 1994) whereas their binding to cellular surfaces has been shown to occur within seconds (Lieske *et al*. 1995).

Crystals adhere to anionic sites on cell surfaces (Bigelow *et al*. 1998, Grover *et al*. 2008). This process is influenced by many factors pertaining to (1) the cells, (2) the crystals and (3) the binding milieu. These are discussed below. The adhesive *properties of cells* can be affected by factors such as cell type, the presence of membrane receptor molecules such as hyaluronan (Zimmerman *et al*. 1999, Asselman *et al*. 2003) and osteopontin (Asselman *et al*. 2003, Verhulst *et al*. 2003), cell polarity (Riese *et al*. 1992, Wiessner *et al*. 2001) and cellular integrity (Wiessner *et al*. 2001, Asselman *et al*. 2003, Verhulst *et al*. 2003), membrane fluidity (Bigelow *et al*. 1997a), the chemical composition and pH of ambient medium (Kasemo and Lausmaa 1994), cell surface electric charge (Lieske *et al*. 1996b), phosphatidyl serine groups (Bigelow *et al*. 1997b, Wiessner *et al*. 2001) and proximity of mineral substance that can alter the 3-dimensional configuration of a cellular protein (Ghosh *et al*. 2007).

Furthermore, the *conformation of proteins on crystal surfaces* is known to influence the binding affinity of the latter. For example, the binding of UPTF1 to CaOx depends on the presence of γ-carboxyglutamic acid residues near its N-terminus (Doyle *et al*. 1991) whereas binding of osteopontin to hydroxyapatite relies on its component glutamic acid and aspartic acid residues (Goldberg *et al*. 2001) as well as the degree of phosphorylation of the protein (Langdon *et al*. 2009).

Ionic composition of the surrounding medium can alter conformation of proteins and therefore their binding potential. For example ambient calcium concentration affects the binding of UPTF1 to COM (Ryall *et al*. 2005), osteopontin to COD (Ryall *et al*. 2005, Thurgood *et al*. 2008) and matrix Gla protein to hydroxyapatite (Roy and Nishimoto 2002) .

Other factors that influence the binding process are: collagen IV, osteopontin and urinary proteins containing sialic acid (Lieske *et al*. 1995, Yasui *et al*. 2002), whereas urinary anions such as glycosaminoglycans (e.g. chondroitin sulphate A and B, heparin sulphate and hyaluronic acid), glycoproteins (e.g. bikunin, THF and nephrocalcin) and citrate can oppose the binding of crystals to cells (Lieske *et al*. 1995, Ebisuno *et al*. 1999a, Kumar *et al*. 2003).

These aforementioned macromolecules have shown their inhibitory effect by altering the macromolecular composition of the binding medium in which crystal attachment is investigated (Lieske and Toback 1993, Ebisuno *et al*. 1999a) or by inducing macromolecular changes to the anionic sites on the cell surface (Verkoelen *et al*. 2000, Yasui *et al*. 2002). However a crucial factor that limits the physiological relevance of these aforementioned studies is the use of inorganic COM (iCOM) crystals which differ in size and morphology from urinary CaOx (uCaOx) crystals. Furthermore urinary COM crystals contain surface-bound and intracrystalline proteins, which iCOM do not, and these proteins affect the adhesion of these crystals (Grover *et al*. 2007, Grover *et al.* 2009). Another limitation of many reported studies is the use of simple aqueous binding media such as PBS, synthetic urine or Tris-HCl, rather than human urine (Lieske and Toback 1993, Lieske *et al*. 1995, Verkoelen *et al*. 2000, Kumar *et al*. 2005). Human urine is a far more complex medium as it contains proteins and other macromolecules and these have been shown to influence crystal-cell adhesion (Grover *et al*. 2007).

Therefore investigations mimicking physiological conditions are warranted. The aim of this investigation was to study and quantify the effect of *Folium pyrrosiae*, *Desmodium styracifolium*, *Hylocerus trigonus*, *Phyllanthus niruri*, *Orthosiphon stamineus* and Cystone® on CaOx crystal binding to MDCK-I cells.

4.2 Methodology

4.2.1 Preparation of plant extract stock solutions

Stock solutions of *Folium pyrrosiae*, *Desmodium styracifolium*, *Hylocerus trigonus*, *Phyllanthus niruri*, *Orthosiphon stamineus* and Cystone were prepared at twice the concentration recommended in chapter 3.2.1. These stock solutions were used in two ways: (1) to precoat CaOx crystals prior to the binding assay and (2) to precoat MDCK-I cells prior to the binding assay. Stock solutions of herbal extracts were prepared at four times the recommended concentration in chapter 3 where the plant extract was present in the medium itself during the binding assay. The stock solution concentrations were chosen to keep the final concentration of plant extracts constant in the respective experiments. These treatment steps will be explained in greater detail in section 4.2.6.

4.2.2 Preparation of CaOx crystals

Two types of CaOx crystals were used in this investigation, namely iCOM and uCaOx. iCOM was commercially obtained (*Sigma Aldrich, Germany*) and its composition was confirmed using XRD (data presented in Appendix 2). uCaOx crystals were prepared from individual twenty-four hour urines of three groups of South African subjects, i.e. healthy black (n=5) and healthy white (n=5) males and white recurrent CaOx male stone formers (n=5). Urines were collected in plastic bottles without preservative. Owing to the rarity of renal calculi in the black population, it was not possible to recruit black stone formers. Creatinine concentrations were measured in all urine samples to ensure their integrity. Urine collections that tested positive for the presence of haematuria or nitrites using urinalysis test strips (*Boehringer Mannheim, Germany*) were excluded from this study. Urine was pre-filtered through a 0.75 μm filter followed by 0.45 μm nitrocellulose filter paper before use.

In order to harvest uCaOx crystals, the MSL of each twenty-four urine sample was measured as described in chapter 2.3. Thereafter, a 500 mL aliquot was allowed to equilibrate at 37 ˚C in a shaking water bath at 100 rpm (*Labcon, Johannesburg, South Africa*). It was dosed subsequently

with 5 mL Na_2Ox at a concentration of 30 mM above the previously determined MSL at 0 minutes and again at 1 hour. After a 2 hour incubation period, crystals were filtered (0.22 μm) and washed thoroughly with large quantities of distilled water and allowed to dry overnight at room temperature. XRD was used to confirm the composition of CaOx. Crystals from urine of each of the three groups were pooled respectively for the crystal binding assay to eliminate intra-racial differences and provide sufficient material for the respective experiments.

4.2.3 Cell culture

Cells were cultured according to the method described by Lieske *et al*. (1992b) under sterile conditions in a laminar flow hood. The working area of the hood was sterilized using 70% ethanol and by exposure to ultra-violet (UV) light for 20 minutes prior to experimental work. Solutions required for cell culture work were made up as follows using sterile water (distilled water filtered through a 0.22 μm membrane).

PBS (phosphate buffered saline) was prepared by adding 11.5 g Na_2HPO_4 (sodium hydrogen phosphate), 2.28g NaH_2PO_4 (sodium phosphate) and 43.84 NaCl (sodium chloride). The pH was adjusted to 7.5 using 1 M HCl and the volume was made up to 5 L with sterile water.

Trypsin-EDTA (ethylenediaminetetraacetic acid) was constituted from 0.18 g trypsin (0.09%) and 0.08 g EDTA (0.5%) in 200 mL PBS. The pH was adjusted to 7.5 using sodium hydroxide (NaOH) and filter sterilized (0.22 μm).

HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) solution (1 M) was made up by adding 59.58 g of the salt to 250 mL sterile water. The pH was adjusted to 7.5 using 5M NaOH and the solution was autoclaved.

Live MDCK-I cells were generously supplied by Dr John Lieske (Department of Medicine, Mayo Clinic, Rochester, USA). Cells were grown in *Dulbecco-Vogt modified Eagle's* medium (DMEM) containing 10% fetal calf serum (FCS), 2% HEPES and 1% penicillin-streptomycin (*BioWhittaker, Walkersville, USA*). DMEM containing the aforementioned constituents at the

given concentrations will be referred to henceforth as 10% DMEM. The culture medium was sterilized using a 0.22 μm filter unit (*VacuCap 90 PF, Roseville, Michigan, USA*) under vacuum.

Upon arrival in the laboratory, the confluent cells were subcultured to prepare stock supplies. Medium was aspirated from the T-225 tissue culture flask (*Nunclon, Denmark*) using a Pasteur pipette and 5 mL PBS was added to the flask. The entire surface was rinsed by rocking the culture plate gently. PBS was aspirated from the culture plate using a Pasteur pipette and this rinsing step was repeated. A minimum volume (2 – 5 mL) of trypsin-EDTA solution was used to cover all of the cells and the flask was placed in a 5% CO_2 incubator (*United Scientific, Oregon, United States*) at 37 ˚C. After 5 minutes, it was removed from the incubator and the flask was tapped repeatedly underneath to facilitate cell detachment (this was necessary as the cells were highly adherent) which was monitored using a light microscope (*ID02, Ziess, Germany*). The detached cells were transferred in the trypsin-EDTA solution into a sterile 15 mL reaction tube containing 10% DMEM and centrifuged (*Centrifuge, Damon, USA*) at 2000 rpm for 5 minutes. Thereafter the supernatant was discarded and the cell pellet was resuspended in 1 mL of 10% DMEM and vortex-mixed (*Vortex-2 Genie, New York*). The 1 mL cell suspension was pipetted into a vial containing 1 mL dimethyl sulphoxide (DMSO) in 8 mL of 10% DMEM. Aliquots (0.5 mL) of the DMSO cell suspension were pipetted into 2 mL cryogenic vials and stored in liquid nitrogen (- 174˚C) until later use in crystal binding experiments.

To prepare cells for the binding assay, two vials of frozen cells were removed from liquid nitrogen and thawed at room temperature. The cells were transferred immediately into a sterile 15 mL reaction tube containing 1 mL DMEM and centrifuged (*Centrifuge, Damon, USA*) at 2000 rpm for 5 minutes. The supernatant was discarded and the cell pellet was resuspended in 1 mL of 10% DMEM and vortex-mixed (*Vortex-2 Genie, New York*). This washing step was repeated. Thereafter, the cell pellet was resuspended in 1 mL of 10% DMEM. The cell suspension was added to a 100 mm sterile plastic culture plate (*Nunclon, Denmark*) containing 9 mL 10% DMEM. The culture plate was swirled gently to distribute cells evenly and the cells were allowed to grow at 37 ˚C in a 5% CO_2 incubator (*Shel Lab, United Scientific, Oregon, United States*). Cell growth was monitored daily by examination of cell morphology under a light microscope (*ID02, Ziess, Germany*). Ideally cells should grow in monolayers with high levels of

differentiation and form junctional complexes, *i.e.* pack closely together. Fresh 10% DMEM was added every 2 – 3 days and confluence was achieved in approximately 5 days.

Once confluent, cells were lifted from a 100 mm plate (as described previously) and split into fifteen 35 mm plates each containing 2 mL DMEM constituted from 5% fetal calf serum (FCS), 2% HEPES and 1% penicillin-streptomycin (*BioWhittaker, Walkersville, USA*). DMEM containing the aforementioned constituents at the given concentrations will be referred henceforth to as 5% DMEM. The 35 mm plates were allowed to grow at 37 ˚C in a 5% CO_2 incubator (*United Scientific, Oregon, United States*) and confluence was usually achieved in 2 days.

4.2.4 Crystal-cell binding assay

Inorganic and urinary crystals were sonicated (*Bandelin Sonorex, Germany*) for 30 minutes to ensure that the crystalline material contained particles of a uniform size. Crystals were added to either sterile water or urine, depending on the experiment, in a 100 mL soda-lime glass flask containing a magnetic stirrer bar (3 cm). This glassware was chosen as it reduces the sticking of crystals to the sides of the flask. Crystal slurries were prepared at a concentration of 4.285 mg/mL and the slurries were allowed to stir overnight at room temperature on a magnetic stirrer (*VELP Scientifica, Europe*) prior to the binding assay. This extended period of stirring ensured that all crystals were in suspension so that the concentration of CaOx used for each experiment remained uniform.

The following solutions were required for the crystal-cell binding assay were made up as follows.

6M HCl (hydrochloric acid) was made up by adding 136 mL concentrated HCl (32%) to 64 mL sterile water to give a total volume of 200 mL.

100 mM PMSF (phenylmethanesulfonylfluoride) was made up by dissolving 0.871 g of the salt in 50 mL ethanol.

Triton (polyethylene glycol p-(1,1,3,3-tetramethylbutyl)-phenyl ether) lysis buffer was constituted from 29.5 mL sterile water, 2.5 mL 1 M HEPES, 12.5 mL 2M NaCl, 0.5 mL Triton X-100 and 0.5 mL 100 mM PMSF to make up to a final volume of 50 mL. The solution was stored at 4 ˚C.

For the binding assay, the medium was aspirated from the 35 mm plates and the cells were washed with 1 mL PBS or urine, depending on the experiment to be performed (see section 4.2.6). An aliquot (1.05 mL) of the same medium used to wash the cells was then added to each culture plate. All subsequent steps were performed under non-sterile conditions.

The crystal slurry (950 µL) was added dropwise (so as to not disturb the cells) to each culture plate to achieve a final CaOx concentration of 2 mg/mL. The plate was agitated gently for 10 seconds and crystals were left in contact with cells for an additional 2 minutes to allow binding to occur. After the 2 minute binding period, unattached crystals and medium were removed. Further washing with PBS was achieved by using a total of 10 mL PBS in a 10 mL plastic pipette which was fitted with a pipette aid to control the flow rate. The culture plate was swirled gently to allow the PBS to wash the surface of the cells and thereafter the PBS was poured off.

Crystal binding was measured by determining the amount of calcium (from the calcium oxalate) that bound to cells. In order to achieve this, cells were lysed overnight by the addition of 600 µL 6 M HCl to each culture plate. Thereafter the lysates were transferred to 15 mL centrifuge tubes. The culture plate was washed with a further 400 µL 6 M HCl followed by two aliquots of 500 µL sterile water. The combined washings were added to the centrifuge tube and the total cell lysate (~2 mL) was centrifuged (*Centrifuge, Damon, USA*) at 2000 rpm for 5 minutes. The supernatant was analysed using an atomic absorption spectrometer (*Varian, SpectAA5, Germany*) to determine the total amount of calcium (bound and endogenous cellular calcium). The pellet was retained for total protein determination.

Total protein in each culture plate was assayed by the Bradford method (Bradford 1976) using bovine serum albumin as the standard (BSA standards, 1 – 10 µg/ml; *Bio-Rad, Germany*). The assay was carried out in a 96-well plate (*Nunclon, Denmark*) and the following volumes were

used: 3 µL cell lysate, 157 µL sterile water and 40 µL Bio-Rad protein assay dye reagent concentrate (*Bio-Rad Lab, Munich*). Absorbance was measured at 595 nm using a spectrometric plate reader (*Anthos 2001, Labtec, Germany*). Each sample was measured in duplicate.

4.2.5 Determination of optimal binding conditions

The concentration of crystal slurry (2 mg/mL) was chosen based on previous work carried out by members of the Kidney Stone Research Laboratory (KSRL) at the University of Cape Town (unpublished data, 2007). In most of the studies investigating crystal-cell binding (referenced in section 4.1), measurements of total calcium bound to cells were made using radio-labelled [^{14}C] CaOx crystals which were counted on a scintillation counter. However, in studies carried out at the KSRL, calcium was determined using an atomic absorption spectrometer. This technique is a less sensitive than the scintillation counter. As such, a greater concentration of calcium was required in binding assays.

Confluent 35 mm cell culture plates contain between 0.8 – 1.0 x 10^6 cells (Kumar *et al*. 2003, Atmani *et al*. 2004). In binding assays, a crystal-cell ratio of 1:1 is considered acceptable (*personal communication: Dr John Lieske, Department of Medicine, Mayo Clinic, Rochester, USA*). In order to determine the number of crystals present in slurry of concentration 2 mg/mL, an aliquot was countered on the Coulter Multisizer II (Coulter Electronics Ltd., England) fitted with a 140 µm orifice (2.8 – 90.0 µm particle size range). The number of crystals counted was 3.2 x 10^5. However the coulter counter does not recognize aggregates and even though the slurry was stirred overnight (not sonicated), this number was an underestimate due to the presence of crystal aggregates. Even if this number is doubled, a concentration of 2 mg/mL crystals is still acceptable based on the crystal-cell ratio given above.

In a crystal-cell binding experiment involving crystal slurries of concentration 0.2, 0.4 and 0.8 mg/mL, a linear increase in adhesion was measured (R^2= 0.999). A further increase in the crystal binding was detected when using slurry of 2 mg/mL. However, crystal slurry of concentration 3.8 mg/mL induced a decrease in binding relative to the 2 mg/mL concentration which could be attributed to ‘over-crowding’ causing a hindrance to the binding process. Therefore 2 mg/mL

was considered the ideal slurry concentration for binding experiments as satisfies the crystal-cell ratio in 35 mm cell culture plates and provides sufficiently high calcium levels which can be detected by atomic absorption spectrometry. In experiments involving MDCK-I cells, a 2 minute period was considered a standard short time to assess crystal attachment which occurs rapidly (Kumar *et al*. 2003, Atmani *et al*. 2004, Lee *et al*. 2009).

4.2.6 Modifications to the binding assay

In order to test the inhibitory properties of the plant extracts, the crystal-cell binding assay was modified in 3 ways which were adapted from studies where the inhibitory activitiy of different macromolecules on crystal-cell binding were investigated. Plant extracts were used to either pre-incubate CaOx crystals (Schepers *et al*. 2002. Kumar *et al*. 2003, Grover *et al*. 2007), precoat MDCK-I cells (Ebisuno *et al*. 1995, Kumar *et al*. 2003, Grover *et al*. 2007) or to be present in the binding medium (Atmani *et al*. 2004). In addition, conditions were chosen to mimic those which occur physiologically. This was achieved by using real urine instead of PBS as the binding medium (Ebisuno 1999b, Grover *et al*. 2007) and also by substituting iCOM for CaOx crystals precipitated from real urine (Grover *et al*. 2009). The details of the various experiments are summarized in Table 4.1.

Table 4.1: Design of crystal-cell binding experiments to investigate the effect of different plant extracts

Crystals:	**iCOM**			**iCOM**			**uCaOx**		
Binding medium:	**PBS**			**Pooled urine**			**PBS**		
Plant extract used:	*To precoat crystals*	*To precoat cells*	*In binding medium*	*To precoat crystals*	*To precoat cells*	*In binding medium*	*To precoat crystals*	*To precoat cells*	*In binding medium*
Folium pyrrosiae	√	√	√	√	√	√	-	√	-
Desmodium styracifolium	√	√	√	√	√	√	-	√	-
Hylocerus trigonus	√	√	√	√	√	√	-	√	-
Phyllanthus niruri	√	√	√	√	√	√	-	-	-
Orthosiphon stamineus	√	√	√	√	√	√	-	-	-
Cystone®	√	√	√	√	√	√	-	-	-

Notation: √ denotes that the experiment was carried out,
- denotes that the experiment was not carried out due to insufficient quantity of CaOx crystals

(i) Precoating of CaOx crystals with plant extracts

iCOM crystals were precoated with plant extract prior to the assay as follows. A 2.5 mL aliquot of plant extract and 2.5 mL binding medium (either PBS or urine) were added to 21.4 mg iCOM, and the mixture was stirred rapidly (*VELP Scientifica, Europe*) for 15 minutes. Thereafter the slurry was transferred to a 15 mL centrifuge tube and centrifuged (*Centrifuge, Damon, USA*) at 2000 rpm for 5 minutes. The supernatant was discarded and the crystals were washed with 3 mL medium. After centrifugation, the supernatant was discarded again. The crystals were resuspended in 3 mL medium and the slurry was set-up and the assay was carried out the following day as described in section 4.2.4.

(ii) Precoating of MDCK-I cells with plant extracts

MDCK-I cells were precoated with plant extract prior to the assay as follows. Cells, which were cultured as described in section 4.2.2, were washed with 1 mL medium on the day of the binding assay. Thereafter 1 mL plant extract and 1 mL 5% DMEM were added to each culture plate and cells were placed in a 5 % CO_2 incubator for 15 minutes at 37 ˚C. Afterwards the medium was

aspirated. Cells were washed with an aliquot of medium (1 mL) and a further aliquot of the same medium (1.05 mL) was added to each plate. The assay continued as described in section 4.2.4.

(iii) Addition of plant extracts to the binding medium

Cells were cultured as described in section 4.2.4. An aliquot (0.5 mL) of the four times concentrated plant extract (prepared as described in section 4.2.1) and 0.55 mL medium were added to each cell culture plate. The combined medium and plant extract was used to bathe cells and crystals during crystal binding. The assay continued as described in section 4.2.4.

(iv) Human urine as the binding medium

Binding assays were carried out in which pooled urine from black and white males was used as the medium in which cells and crystals were bathed. Twenty-four urines were collected from healthy black (n=5) and white (n=5) males. Urine exclusion criteria were described earlier in section 4.2.2. Pooled urines for each race group, constituted from 200 mL aliquots from each male, were pre-filtered through a 0.75 μm filter followed by 0.45 μm nitrocellulose filter paper before use.

(v) Urinary CaOx crystals instead of iCOM

In these binding assays, PBS was used as the medium in which cells and crystals were saturated. Crystal slurries were made up of urinary CaOx crystals in PBS as described in section 4.2.4. These crystals were precipitated from the urine of healthy black and white males and white CaOx stone formers as described in section 4.2.2. Owing to a limited quantity of urinary crystals, it was not possible to apply all modifications to the binding assay described in section 4.2.10 thus only pre-coating of cells was carried out. Three plant extracts were selected for investigation namely *Folium pyrrosiae* (the principal plant of interest in this thesis purported to be effective in the treatment of urolithiasis), *Desmodium styracifolium* (another traditional Chinese remedy that demonstrated strong inhibitory activity in *in vitro* studies) and *Hylocerus trigonus* (a plant for which there is no published data). Binding assays were carried out as described in section 4.2.4.

4.2.7 Statistical analysis

Experiments were carried out in triplicate and average values are reported. Data were statistically analyzed by *Graphpad Instat* and considered statistically significant when $p \leq 0.05$.

4.3 Results

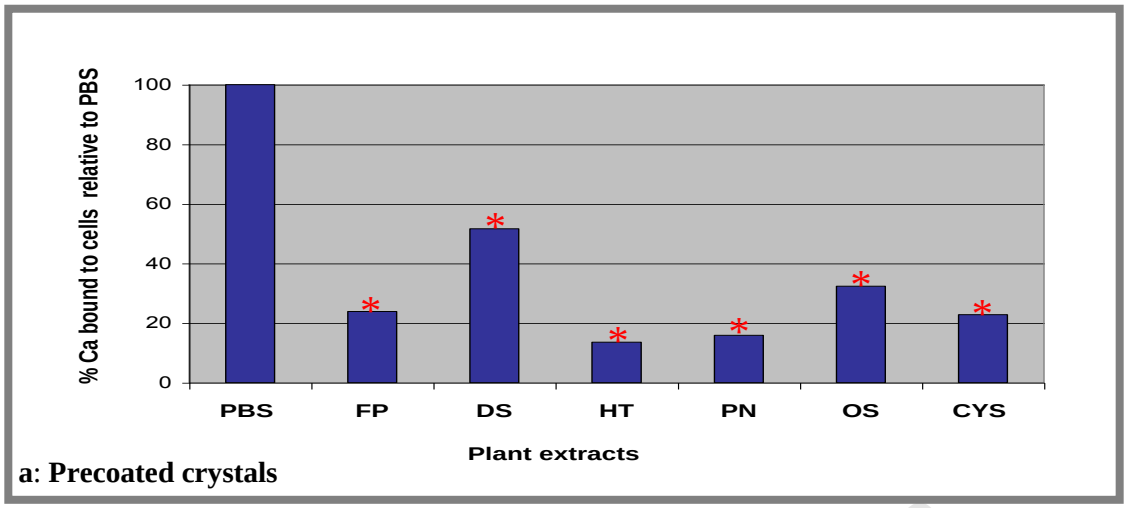
All investigations summarized in Table 4.1 were carried in triplicate and at the end of each binding experiment and the total amount of calcium in each cell lysate in each plate was determined in duplicate. Hence each result presented in the respective graph is the average of 6 measurements. Endogenous cellular calcium was measured in confluent 35 mm cell culture plates (blank reading: cells incubated in water alone) and this concentration was subtracted from total calcium assayed (calcium from cells and bound crystals) in order to obtain the net amount of calcium from the bound crystals. This value measured in the control i.e. iCOM binding to MDCK-1 cells in PBS in the absence of plant extracts, was set at 100%. **All other measurements of bound calcium are presented relative to the control. This was done for the sake of clarity and to allow relative comparisons i.e. between modified experiments**.

The following colour scheme was employed when presenting data of experiments carried out in different binding media i.e. PBS, BU and WU by blue, black and white bar graphs respectively. Protein measurements were carried out in each culture plate to reflect the number of cells present. Raw data of all calcium and protein measurements are reported in Appendix 2.

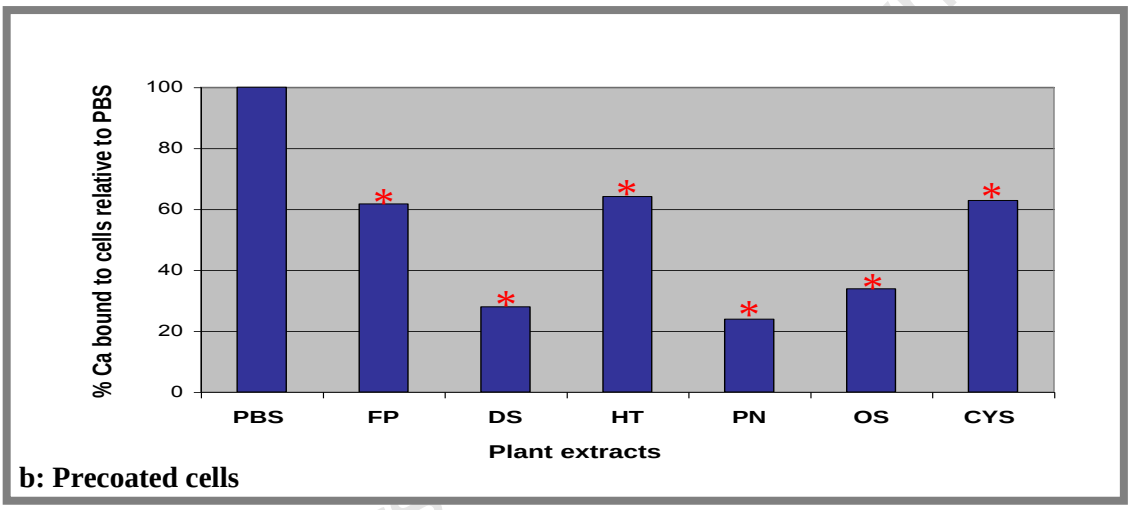
4.3.1 Crystal-cell binding of iCOM in PBS

The binding of iCOM crystals to MDCK-I cells in PBS and the effect of the plant extracts on this process are illustrated in Figures 4.1a-c. The results are presented relative to the PBS control which has been set to a 100%. When plant extracts where used to precoat crystals, there was a significant decrease ($p<0.05$) in crystal-cell binding (Figure 4.1a). A similar effect i.e. a significant decrease in crystal-cell binding was bought about when plant extracts were used to pre-coat cells (Figure 4.1b). Plant extracts present in the PBS binding medium significantly decreased ($p<0.05$) the crystal-cell binding (Figure 4.1c).

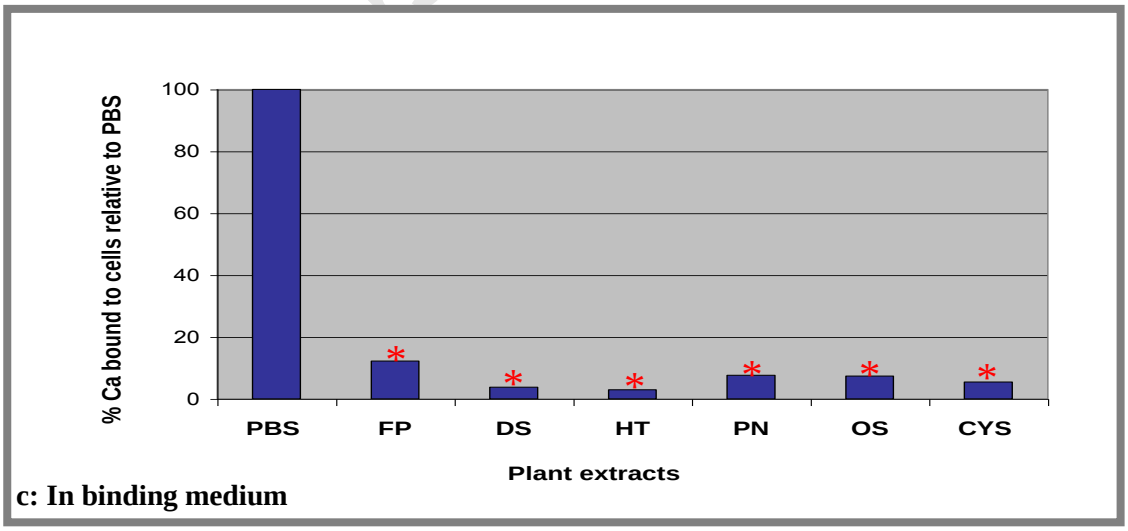
Phyllanthus niruri proved to be the strongest inhibitor overall statistically (based on p values) and superior inhibitory activity in PBS was also demonstrated by *Desmodium styracifolium* and *Orthosiphon stamineus*, compared to the other plant extracts, when present in medium. There were no other statistically significant differences in the relative strengths of the plant extracts (based on p values). Raw data of all measurements are reported in Appendix 2.



a: **Precoated crystals**



b: Precoated cells



c: In binding medium

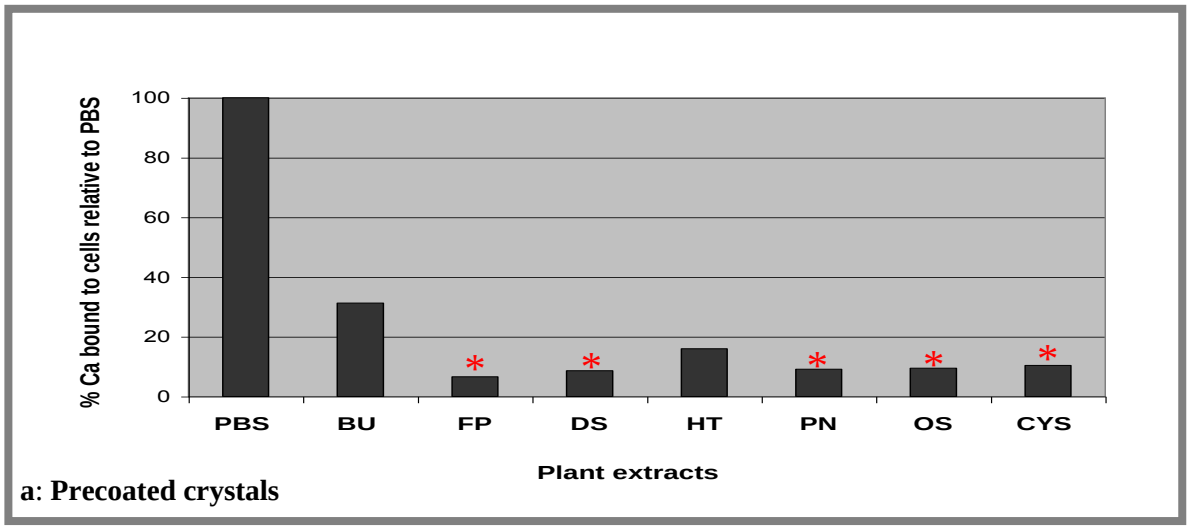
Figure 4.1: The average percentage of calcium bound to MDCK-I cells in PBS where plant extracts were used to (a) to precoat crystals, (b) precoat cells or when (c) present in binding medium. Asterisks indicate $p<0.05$, relative to the PBS control

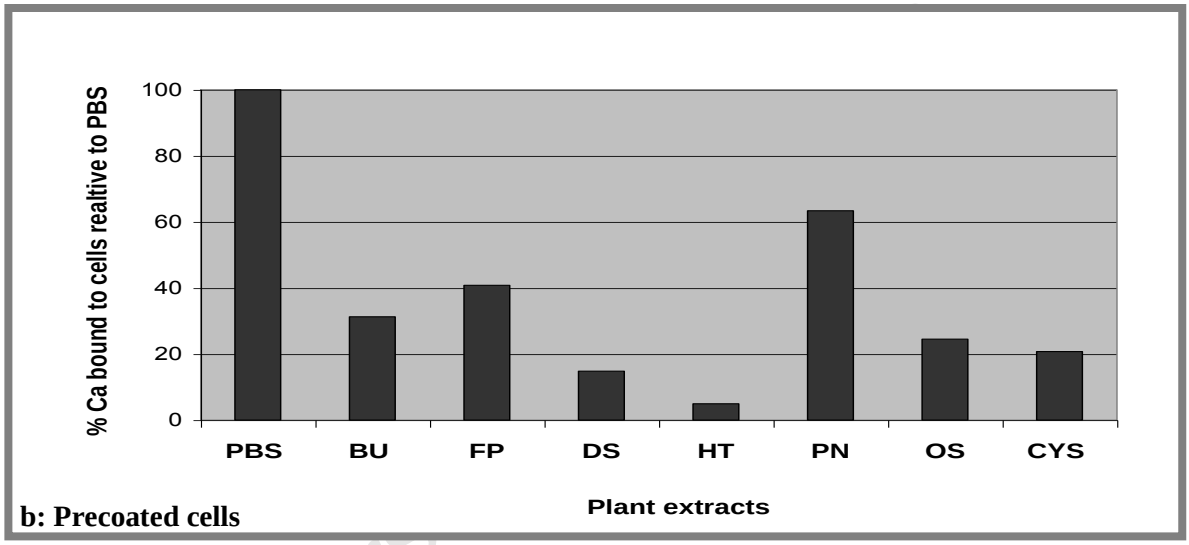
4.3.2 Crystal-cell binding of iCOM in urine

Studies in pooled urine from black males (BU)

The effect of the plant extracts on iCOM crystals binding to MDCK-I cells incubated in BU are illustrated in Figures 4.2a-c. The data are presented relative to the PBS control (the same value as in Figure 4.1 to enable relative comparisons); however **statistical comparisons in this section are presented relative to the BU control** (i.e. where crystal binding was measured in the absence of any plant extract).

All plant extracts significantly decreased ($p<0.05$) crystal-cell binding when used to pre-treat crystals except *Hylocerus trigonus* relative to the BU control (Figure 4.2a). The plant extracts showed no significant effect when used to pre-treat cells prior to binding relative to the BU control (Figure 4.2b). And all plant extracts, when present in the binding medium, significantly decreased ($p<0.05$) crystal-cell binding (Figure 4.2c) relative to the BU control. However there were no statistically significant differences in the relative strengths of the plant extracts. Raw data of all measurements are reported in Appendix 2.





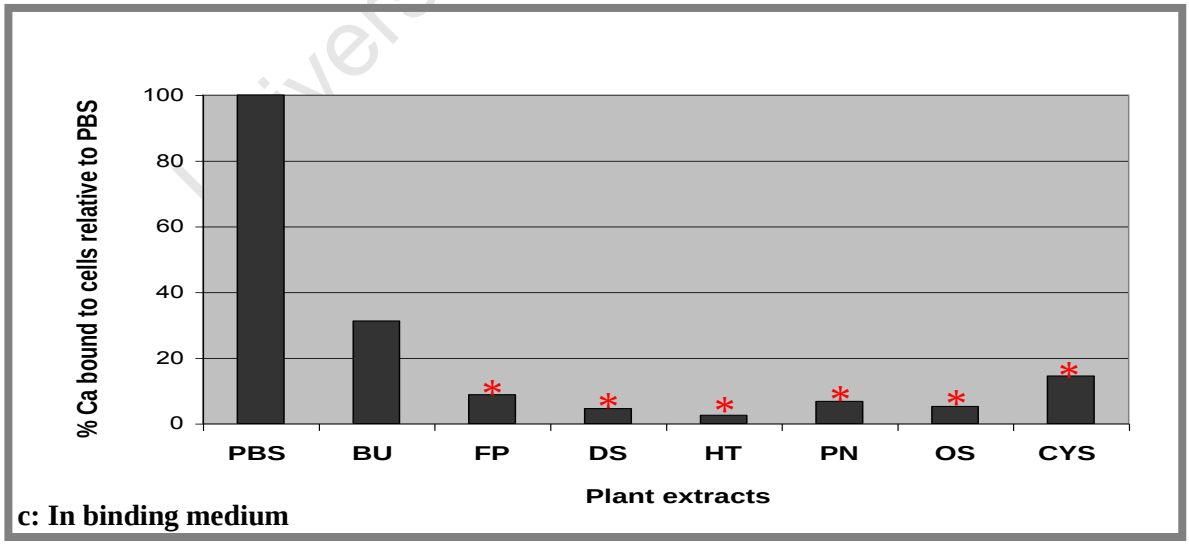
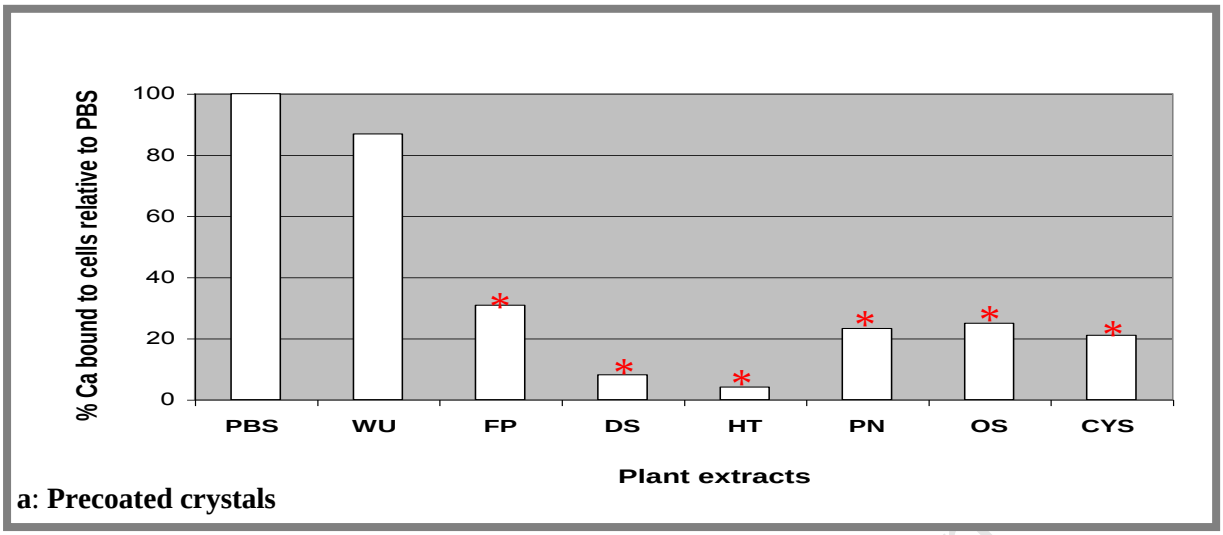


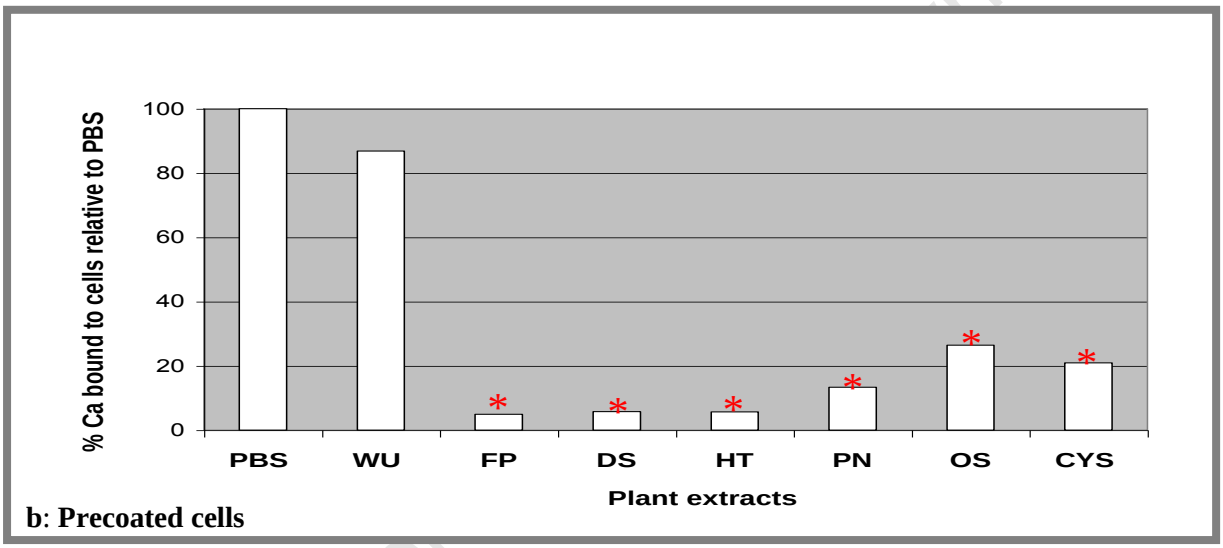
Figure 4.2: The average percentage of calcium bound to MDCK-I cells in PBS where plant extracts were used to (a) to precoat crystals, (b) precoat cells or when (c) present in binding medium. Asterisk indicate $p<0.05$, relative to the BU control

Studies in pooled urine from white males (WU)

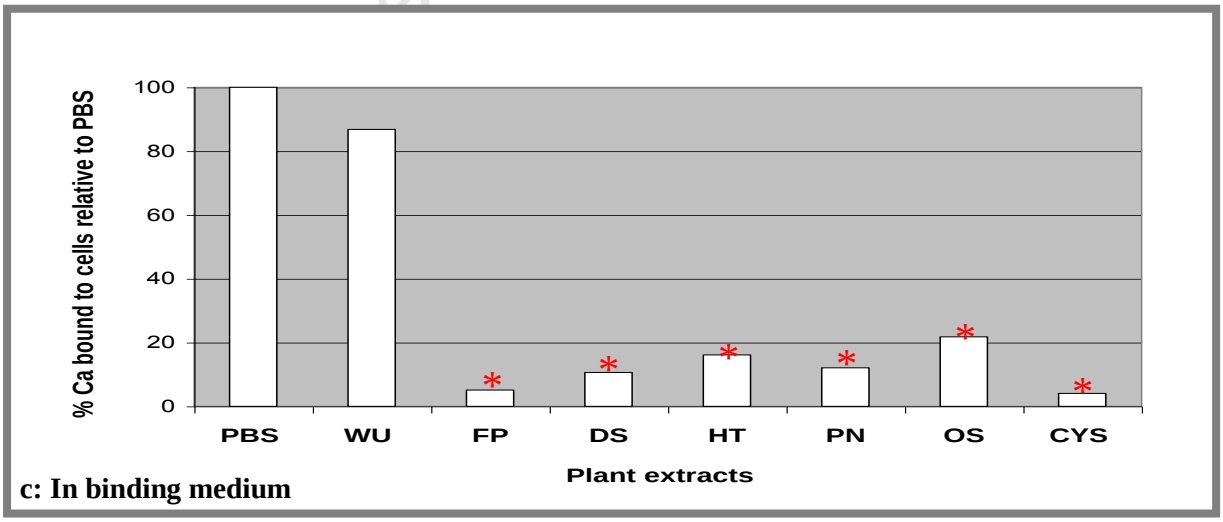
The effect of the plant extracts on iCOM crystals binding to MDCK-I cells in WU are illustrated in Figures 4.3a-c. **Statistical comparisons are made relative to the WU control** (i.e. crystal-cell binding experiment in WU with no plant extract used in the experiment) but as was implemented in BU studies, data are presented relative to the PBS control. All plant extracts significantly decreased crystal binding, whether they were used to pre-treat crystals, pre-treat cells or when present in the binding medium ($p<0.05$). However there were no statistically significant differences in the relative strengths of the plant extracts. Raw data of all measurements are reported in Appendix 2.



a: Precoated crystals



b: Precoated cells



c: In binding medium

Figure 4.3: The average percentage of calcium bound to MDCK-I cells in PBS where plant extracts were used to (a) to precoat crystals, (b) precoat cells or when (c) present in binding medium. Asterisk indicate $p<0.05$, relative to the WU control

4.3.3 Binding of iCOM in different media

The binding of iCOM crystals to MDCK-I cells was measured in PBS, BU and WU alone (Figure 4.4) in order to establish whether binding media affected this process differently. These data are the control values in the experiments presented earlier (Figures 4.1 – 4.3). There was no significant difference in crystal-cell binding in PBS and WU. However, binding in BU was significantly lower ($p<0.05$) than in both of the other two media. Raw data of all measurements are reported in Appendix 2.

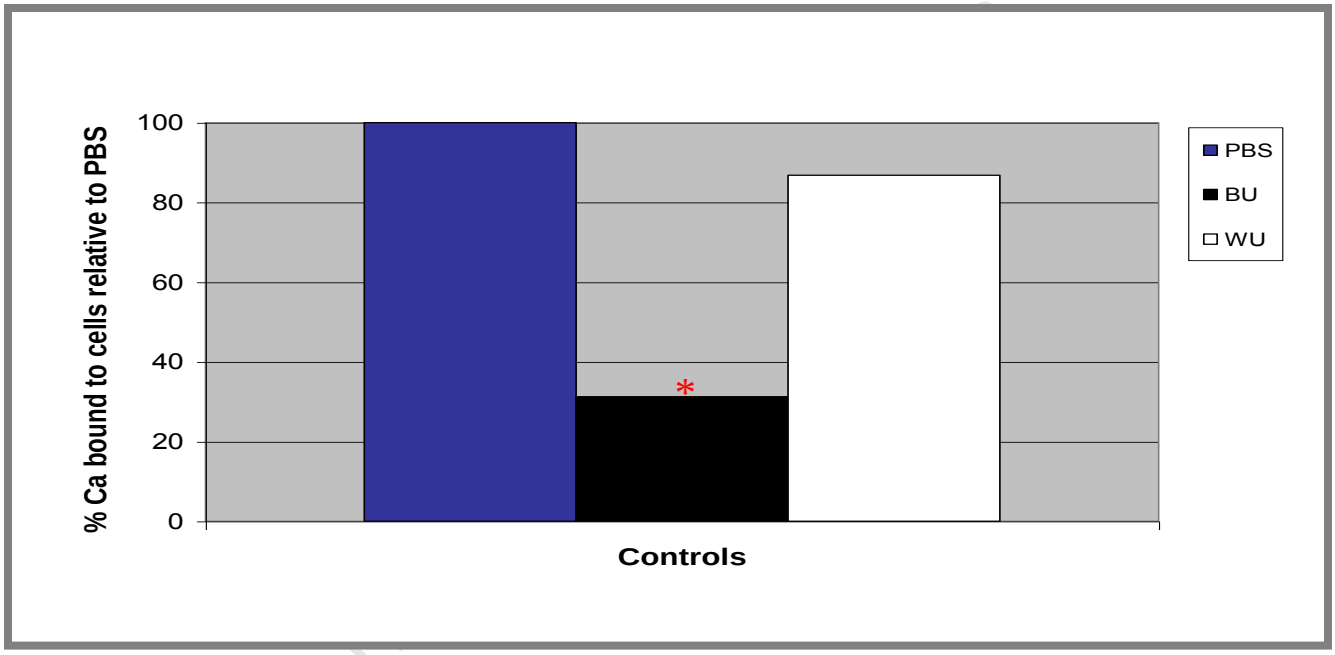


Figure 4.4: The average percentage of iCOM bound to MDCK-I cells in PBS, BU and WU. The asterisk indicates $p<0.05$

4.3.4 Crystal-cell binding of urinary CaOx crystals in PBS

Urinary CaOx crystals were obtained from three sources, namely black (BC) and white (WC) healthy subjects and white recurrent CaOx stone formers (WSF), as described earlier. It was not possible to recruite black stone formers for this investigation. The results are given in Figure 4.5.

Data are presented relative to a control where the assay was carried out in PBS using iCOM crystals (same control value as in Figures 4.1 – 4.3) to allow relative comparisons to be made. However, statistical comparisons were made **relative to the respective urinary crystal control**.

As was mentioned earlier, three plant extracts were selected for investigation in this section, namely *Folium pyrrosiae* (the principal plant of interest in this thesis), *Desmodium styracifolium* (another traditional Chinese remedy that demonstrated strong inhibitory activity in *in vitro* studies) and *Hylocerus trigonus* (a plant for which there is no published data).

All three plant extracts significantly decreased ($p<0.05$) the attachment of urinary crystals BC and WC (*****; *compare bars of the same colour with the control of that colour*). However they showed no effect on the binding of urinary crystals obtained from WSF.

With respect to the controls, urinary crystals bind significantly less ($p<0.05$) to MDCK-I cells than iCOM. It is also noteworthy that urinary crystals from black subjects and urinary crystals from WSF bind significantly less ($p<0.05$) than urinary crystals from white subjects. There were no significant differences in the relative strengths of the plant extracts. Raw data of all measurements are reported in Appendix 2.

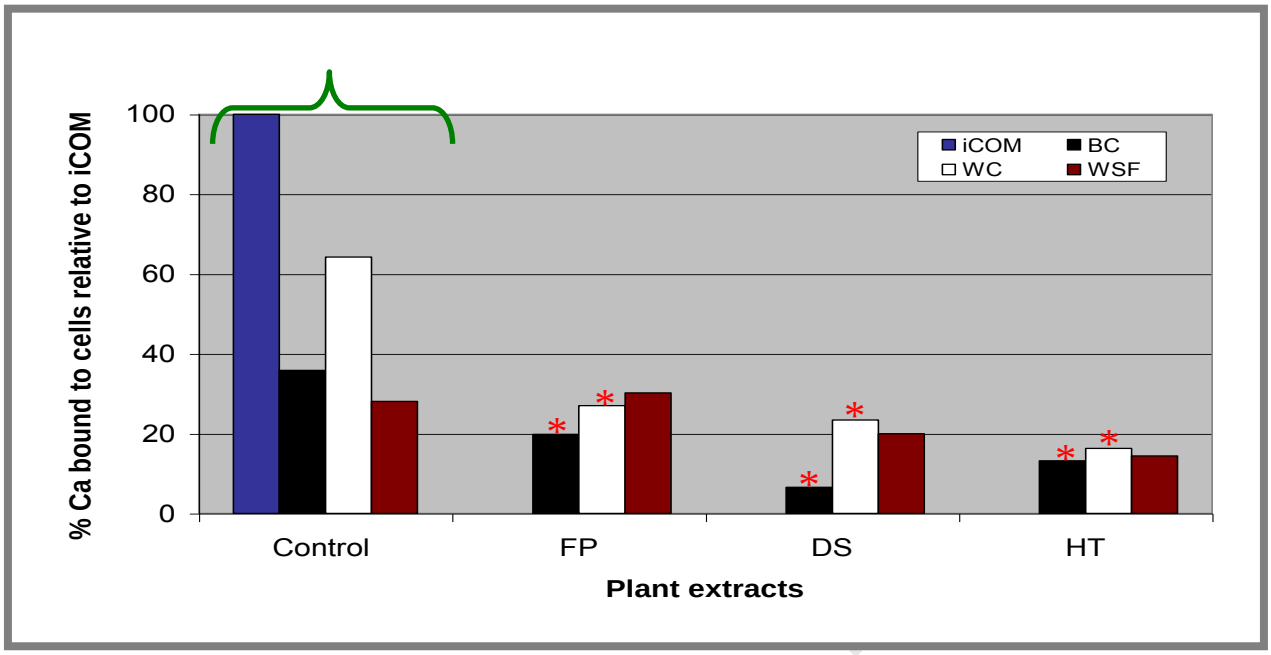


Figure 4.5: The average percentage of calcium bound (iCOM and uCaOx) to MDCK-I cells in PBS. Plant extracts were used to treat cells prior to the binding assay

4.4 Discussion

The binding of a crystal to a cell is considered to be a critical step that triggers a cascade of processes eventually resulting in the genesis of a renal calculus. The initial stage of binding is a surface-controlled phenomenon. However the later stages require complex stereospecific interactions involving a multitude of cytoplasmic, transmembrane and extracellular proteins which synergistically provide a stable point of crystal attachment (Hanein *et al*. 1993, Hanein *et al*. 1994, Grover *et al*. 2009).

Numerous factors influence interactions between crystals and cells, as was explained in section 4.1. It has been suggested that an effective method of inhibition of this process is to neutralize potential binding sites on crystal and cell surfaces (Verkoelen *et al*. 1997). Other inhibitory agents have been found to affect endocytosis by neutralizing potential cell binding sites (i.e. an inhibitor competitively binds to binding sites) thereby facilitating the elimination of crystals in urine (Lieske *et al*. 1995, Verkoelen *et al*. 1995). Therefore inhibitory agents can act in three ways i.e. on the crystal surface, cell surface or by being present in the medium.

In this study, after a 2 minute binding time, all plant extracts under investigation demonstrated inhibitory activity on crystal-cell attachment in some way. In experiments carried out in PBS (involving the use of iCOM), plant extracts were effective in decreasing binding ($p<0.05$) when used to either pre-treat crystals, pre-treat cells or when present in the binding medium. Comparison of Figures 3.1a-c shows that the greatest inhibition of binding occurred when the plant extract was present in the binding medium. This would suggest that the possible mode of inhibition of the plant extracts is competitive binding to the anionic sites on cellular surfaces. This mechanism of inhibition has been reported for other agents mentioned in section 4.1.

In BU, when used to pre-treat crystals and when present in the binding medium, all plant extracts significantly decreased ($p<0.05$) crystal-cell binding (except *Hylocerus trigonus* when used to treat iCOM crystals). However no significant change in crystal-cell binding was demonstrated when plant extracts were used to pre-treat cells prior to binding assays. Similar activity of the plant extracts was demonstrated in WU as compared to PBS where the plant extracts were

effective in decreasing crystal-cell binding ($p<0.05$) in all assays. Plant extracts show greater inhibitory effect in WU compared to BU.

Comparison of the controls in Figure 5.4, where iCOM binding to MDCK-I cells in three types of media was measured, shows that attachment in BU is significantly lower ($p<0.05$) relative to attachment measured in PBS and WU. Previous studies have demonstrated the diverse anions present in whole urine (as mentioned in section 5.1) can coat CaOx and inhibit their binding to cells (Lieske *et al*. 1995, Ebisuno *et al*. 1999a, Kumar *et al*. 2003). This action has been demonstrated here to be greater in BU compared to WU thus BU naturally inhibits the crystal-cell binding process which is considered a risk factor in stone formation. This result is in consensus with the rarity of stones in black subjects. Studies conducted at the KSRL on urinary proteins of black and white healthy male subjects have found a significantly higher ($p<0.05$) total protein in black males (Webber, PhD thesis) and proteins are known to act as inhibitors. However, qualitative analysis of urinary proteins isolated from the two race groups has not yet been accomplished which could assist in explaining the superior inhibitory nature of BU.

Upon comparison of the relative strengths of the plants extracts in sections 4.3.1 and 4.3.2, *Phyllanthus niruri* proved to be the strongest inhibitor overall statistically (based on the p-value), in experiments performed in PBS. Statistically superior inhibitory activity in PBS was also demonstrated by *Desmodium styracifolium* and *Orthosiphon stamineus* when present in medium.

Even though *Hylocerus trigonus* did not exhibit significant inhibitory action on BU when used to pre-treat crystals or cells, it was the strongest ($p<0.05$) inhibitor of cell attachment when present in the binding medium (BU) compared to the other plant extracts. In all other experiments (sections 4.3.1 and 4.3.2) there were no significant differences in the relative potency of the plant extracts.

Plants contain phytochemicals which are biochemically active chemical compounds (Lui 2003). These are generally non-essential nutrients but have been proven to provide great medicinal benefits. Different classes of phytochemicals have been identified namely *phenolics* (e.g. flavonoids, ligands), *terpenes* (e.g. saponins, carotenoids, lipids), *betalains*, *organosulfides* (e.g.

isothiocyanates, allium), *indoles*, *protein inhibitors* and *organic acids* (e.g. oxalic acid, phytic acid) (Shahidi and Naczk 1995, Lui 2003). A few examples of each class are provided here but more than 5 000 phytochemicals are known (Lui 2003).

As mentioned in chapter 1.5.4, phenolics and terpenes are been identified in all plants under investigation in this study, with the exception of *Hylocerus trigonus* (for which there is no published data). Phytochemicals are known for their antioxidant and anticancer properties (Ames and Gold 1991, Milder *et al*. 2005, Thakur *et al*. 2011, Thoppil *et al*. 2011). But saponins, in particular, are known to affect the permeability of cellular membranes but disrupting the lipid bilayer (Cohen *et al*. 1996, Baumann *et al*. 1999). As mentioned above, binding is a surface-controlled phenomenon. Hence changes to the cellular membrane caused by saponins would impact this process and this may be the mode of action of the different plant extracts. The actual mechanism by which saponins act is unclear.

Comparison of the binding of iCOM and uCaOx to MDCK-1 cells in PBS is depicted in Figure 4.5. Crystals isolated from the urine of healthy black and white males and white stone-formers exhibit significantly lower ($p<0.05$) binding affinity compared to iCOM. It is also noteworthy that the binding capacity of crystals decreases significantly ($p<0.05$) in the following sequence iCOM < WC < BC < WSF. Regarding the observation that uCaOx crystals bind to renal epithelial cells significantly less than iCOM, uCaOx differ in size and morphology from iCOM, uCaOx crystals contain surface bound and intracrystalline proteins.

In the preparation of uCaOx, urine was filtered twice (0.75 and 0.45 μm) to remove cellular debris and larger macromolecules, prior to crystallization being induced. Even though urinary protein was not quantified in this experiment, a previous study reported that centrifugation (10 000g) and filtration of urine through a 0.22 μm decreased the protein content of whole urine from 47.3 to 24.8 mg/mL (Grover *et al*. 2009). Therefore, protein was certainly present in urine when crystallization was induced in this experiment (as a membrane with a larger pore size was used in filtration) and these proteins were incorporated into urinary crystals. As mentioned in section 4.2.2, crystals were washed extensively with distilled water and not NaOH after

incubation therefore some surface bound proteins would also be present (Grover *et al*. 2009, Thurgood *et al*. 2012).

The inclusion of proteins into crystals is known to disrupt their atomic structure and alter the texture of their mineral phase (Aizenberg *et al*. 1995). This effect could extend to the surface of crystals and influence its adhesive properties, by causing changes to surface charge and topology (Grover *et al*. 2009), thereby hampering the binding process. And an inverse relation has been reported between crystal binding and the concentration of protein in the medium from which they were precipitated (Aizenberg *et al*. 1997). Therefore urinary crystals, due to the presence of surface bound and intracrystalline proteins, have a decreased binding affinity for renal epithelial cells than iCOM (reasons given above) and this result is depicted in Figure 4.5. Urine from black subjects is known to have a higher concentration of proteins compared to white subjects and crystals isolated from black urine bind with significantly less avidity those from white urine. This result highlights a possible difference in renal handling between the two race groups and the significant role of proteins on crystal-cell binding.

Decreased binding of crystals from black and white controls (BC and WC) is promising in that the extracts achieve exactly what would be required of them as therapeutic agents. Unfortunately, this prognosis was not confirmed in the crystals from WSF. It is not possible to explain this unexpected observation in terms of the results of the experiments described here. However, it can be speculated that the crystals derived from the urine of WSF have some property (not yet identified) which compromises the effects of the extracts. A shortcoming of the current study is the lack of quantitative information regarding the composition of the uCaOx. XRD analysis of uCaOx revealed a mixture of COM and COD; however the respective amounts are unknown. As reported earlier in this thesis, COD has a decreased binding affinity for renal cells relative to COM. The presence of a high percentage of COD could be a contributing factor to the decreased binding (relative to BC and WC) observed in WSF. The effect of the plant extracts, if any, on the binding of COD has also not been established.

This study has investigated several synergistic variables in an attempt to gain insights into the possible effects of various herbal extracts on the binding of CaOx crystals to renal epithelial

cells. Two different CaOx crystals types were used i.e. those synthesized from a simple inorganic solution and those derived from human urine. In the latter case, crystals were derived from three different human sources namely black and white controls, and white stone formers. Three different possible effects on binding capacity by the herbal extracts were addressed: pre-treatment of crystals, pre-treatment of cells and presence of the extract in the binding medium. Three different binding media were also used i.e. PBS and urine from black and white healthy male subjects.

Very interesting results were obtained. Firstly, all of the plant extracts demonstrated a capacity to inhibit crystal-cell binding (iCOM) via all three routes in PBS and WU. In BU, no effect was detected by any of the plant extracts when used to pre-treat cells, and Hylocerus trigonus showed no effect when used to pre-treat crystals. Secondly, in the experiments involving uCaOx, all 3 plant extracts that were tested (*Folium pyrrosiae*, *Desmodium styracifolium*, *Hylocerus trigonus*) decreases the binding of crystals from BC and WC, but had no effect on the binding of crystals from WSF.

These results demonstrate that these herbal extracts are all potentially useful as therapeutic agents in the treatment of urolithiasis by virtue of their ability to inhibit crystal cell binding which has been identified as one of the most important step in stone formation. Further studies in this area are warranted.

The effect of *Folium Pyrrosiae* on calcium oxalate kidney stone risk factors: an *in vivo* study on healthy South African black and white males

5.1 Introduction

The use of herbal remedies for the treatment of urolithiasis has been practiced since long before the use of Western medicine (cited by: Gohel *et al*. 2006). Since the implementation of the Chinese medicine ordinance in 1999, which gave Chinese medicine practitioners recognition as medical professionals, there has been an emergence of TCM hospitals and an increase in the manufacture of TCM. This trend has extended beyond the Far East to many Western countries, including the United Kingdom where more than 600 TCM clinics are in existence (McNamara and Ke 1995).

According to the principles of TCM, renal stone disease can be attributed to 3 imbalances in the body, i.e. "sha-lin" (strangury from urolithiasis), "shi-lin" (strangury caused by urinary calculus) and "xue-lin" (strangury complicated by haematuria). Herbal remedies for renal stone disease are specifically selected to promote the circulation of "qi", vital energy that is also known as *yang* and induce diuresis which aids stone removal (cited by: Gohel *et al*. 2006).

Herbal remedies are being considered increasingly as a suitable long-term treatment for renal dysfunction (Atmani *et al*. 2000). However some greatly needed scientific backing on their pharmacodynamics is required. An example is *Folium pyrrosiae* which is used commonly in the Chinese medicinal system for the treatment of urolithiasis (Gohel *et al*. 2006). Usually, these herbal blends are prepared by traditional water extraction methods (Zhao 2005). Three independent herbalists suggest a daily dose of 1.5 g of dried herb granules to treat urinary stone problems and in extreme cases, 40-60 g per day can be used in infusions (Zhao 2005). There have been no known cases of toxicity. However mild side-effects such as dizziness, elevated urinary volume and increased hunger have been reported when very high doses are ingested but these symptoms ceased when the treatment was terminated (Zhao 2005).

The primary objective of the study described in this chapter was to investigate the effects of *Folium pyrrosiae* on the physicochemical risk factors for CaOx kidney stone formation. A secondary objective was to determine whether any effects (if they did indeed occur), were different in subjects from South Africa's black and white population. Finally, it was anticipated that the study would provide an opportunity to compare baseline parameters in the two race groups, albeit that this was not the main motivation for undertaking the investigation in the first place.

5.2 Materials and methods

5.2.1 Study design

A double-blind, 7 day randomized, placebo-controlled, cross-over design study was conducted with a 1 week washout period observed between protocols. Subjects were recruited during the period May – July 2009. The cross-over study extended over 5 weeks. *Folium pyrrosiae* was purchased in granular form (*Nong's Powder*). Starch powder (*White Wings* plain wheat flour made in Australia) was used as the placebo and was purchased from a supermarket in Hong Kong by Dr Mayur Danny Gohel (*Department of Health Technology and Informatics, Hong Kong Polytechnic University*). These products were encapsulated by a pharmaceutical company upon instruction of Dr Mayur Danny Gohel and sent via courier to the University of Cape Town.

Healthy black (n=9) and white (n=9) male subjects were recruited from the student cohort at the University of Cape Town. The sample size required to achieve a statistical power of 70% was calculated at a significance level of 0.05 using the computer program *Graphpad Statmate 2* and was based on an average standard deviation of urinary calcium observed in the Kidney Stone Research Laboratory. The protocol for this investigation was approved by the Research Ethics Committee of the University of Cape Town.

5.2.2 Subjects

South African black and white males aged between 18 and 30 years old, with no prior history of clinical or metabolic problems, were allowed to participate in the trial. Diabetic patients were excluded as the capsules enclosing the plant extract and placebo were sugar-based. Prior to the commencement of the study, each subject was required to fill out a questionnaire giving information about their social and medical history (Appendix 3). Subjects followed an unrestricted diet but were advised to avoid consumption of calcium and oxalate rich foods during the trial period. They were also required to keep a record of all food and liquids consumed on urine collection days.

Subjects were solicited via advertisement. An information sheet was provided to them with instructions on how to collect 24 hour urine. They were instructed not to take any supplements or consume any alcohol as these may affect oxalate excretion or constituents of

the traditional herbal medicine which would confound the results of the study. After a verbal and written explanation, they were required to sign a letter of consent. Participation was voluntary and subjects were able to withdraw at any time without prejudice. All information provided was kept confidential. This study was covered by UCT's no-fault liability insurance cover and participants could be compensated in the event of any harm being done to them; however no such incidences were reported.

5.2.3 Protocol

Subjects were administered with 3 capsules, each containing 0.5 g *Folium pyrrosiae*, which they consumed daily after breakfast for 7 days. The same subjects participated in the cross-over study during which 3 placebo capsules containing 0.5 g starch were administered each morning after breakfast for 7 days. Twenty-four hour urine samples were collected on days 0 (baseline), 7 (post treatment) and 14 (washout) of both protocols. Endogenous micronutrient concentration in a daily dosage of *Folium pyrrosiae* [*Calcium = 1.38 mg, Citrate = 6.00 mg, Magnesium = 2.88 mg, Oxalate = 0.55 mg*] and starch [*Calcium = 0.54 mg, Citrate = 1.20 mg, Magnesium = 1.15 mg, Oxalate = 0.32 mg*] were determined by Gohel *et al*. (2006) and were considered too insignificant to interfere with urine chemistry.

Twenty-four hour dietary information was recorded on urine collection days. Baseline creatinine and liver function tests were performed on all subjects on days 0 and 3, as a safety measure i.e. these parameters must not become elevated. Liver function was monitored by means of blood tests for albumin, bilirubin, international normalized ratio (INR) and transaminases. Blood was drawn by specialist phlebotomists and testing was performed by Dr Davies Pathology Laboratories, Cape Town, South Africa. Blood analyses showed no changes in the concentration of liver enzymes (safety-markers) and no side-effects were reported by any of the subjects either.

5.2.4 Experimental methods

Urine collection and analysis

Twenty-hour urine samples were collected in plastic bottles without preservative. Each sample was tested for haematuria and nitrite using urinalysis test strips. All samples were assayed for the following urinary variables: *pH, calcium, citrate, chloride, creatinine, magnesium, oxalate, potassium, phosphate, sodium* and *urate* as described in Chapter 2.3.

The TRI was determined using standard urinary variables and specific gravity was measured using a densitometer (*DMA 3S, Digital Densitymeter, Austria*). Relative urinary supersaturations of CaOx, brushite and uric acid were calculated using EQUIL 2.5. All raw data is presented in Appendix 4.

Crystallization experiments

The CaOx MSL, PSD, rates and inhibition of CaOx crystal nucleation and aggregation as well as [^{14}C]-oxalate deposition were measured in all urine samples both pre- and post-treatment. These methods have been described fully in Chapter 2. All raw data is presented in Appendix 4.

Compliance test

A compliance test was carried out where subjects were instructed to consume a daily dosage of *Folium pyrrosiae* capsules in the presence of the investigator and the corresponding twenty-four urine was collected in 3 aliquots (i.e. at 2 hours, 4 hours and 24 hours after consumption). The samples were assayed for standard urinary variables and their ratios compared as a function of creatinine.

Statistical analysis

Data were analyzed by using *GraphPad Instat* and $p<0.05$ was regarded as significant. Average values and standard error (SE) have been reported. Error bars have been omitted in graphs for the sake of clarity.

5.3 Results

5.3.1 Dietary analysis

The nutrient intake of the two race groups was derived from the analysis of dietary questionnaires which subjects were required to complete on each urine collection day. These were analysed for macro- and micronutrients using the computer program *Foodfinder*^{TM} *2* (Wolmarans *et al*. 2001). The values presented in Table 5.1 are an average of each nutrient on collection days 0 and 7 of the two protocols. There were no significant differences in nutrient intake between the two race groups thereby ruling out the possibility of any confounding dietary factors.

Table 5.1: Mean daily intake of nutrients (SE) for black and white subjects on days 0 and 7 of both protocols

	Blacks	Whites
Moisture (g)	610 (57)	517 (47)
Total protein (g/day)	55.6 (7.7)	40.5 (7.4)
Total fat (g/day)	47.4 (7.4)	43.1 (9.0)
Total sugar (g/day)	15.4 (3.9)	8.71 (1.34)
Carbohydrate (g/day)	155 (19)	104 (11)
Fibre (g/day)	11.6 (1.8)	7.35 (1.31)
Oxalate (mg/day)	50.5 (33.4)	41.1 (26.9)
Calcium (mg/day)	303 (51)	321 (67)
Magnesium (mg/day)	181 (26)	121 (19)
Phosphorus (mg/day)	723 (96)	640 (142)
Potassium (mg/day)	1412 (175)	1015 (150)
Sodium (mg/day)	1267 (234)	1147 (321)
Vitamin A (RE/day)	221 (44)	297 (70)
Vitamin B_6 (mg/day)	1.24 (0.3)	0.78 (0.15)
Vitamin C (mg/day)	119 (31)	41.3 (20.1)
Vitamin D (µg/day)	3.11 (0.9)	1.52 (0.51)
Vitamin E (mg/day)	4.99 (1.40)	4.13 (1.25)

p>0.05 for all nutrients

5.3.2 Urine composition

No changes were detected in urinary variables in the compliance test which confirmed the authenticity of the data from the trial and placebo protocols. Compliance test data are presented in Appendix 4.

Urinary parameters and computed risk indices for both race groups during supplementation with the placebo and *Folium pyrrosiae* are presented in Tables 5.2a and b respectively.

No significant changes were detected in any of the variables during supplementation with the placebo in either race group ($p>0.05$). However, upon comparison of baseline (and washout collection days), urinary calcium and urinary phosphate were found to be consistently higher in white subjects than in blacks ($p<0.05$).

Table 5.2a: Mean urinary parameters (SE) in black and white subjects before and after supplementation with starch placebo and washout

Variables	**BLACKS (n=9)**			**WHITES (n=9)**		
	Baseline day 0	**Post-treatment day 7**	**Washout day 14**	**Baseline day 0**	**Post-treatment day 7**	**Washout day 14**
pH	5.98 (0.21)	6.08 (0.12)	6.12 (0.16)	6.08 (0.11)	6.11 (0.09)	6.11 (0.13)
Volume (mL/24 hr)	1573 (158)	1280 (160)	1220 (175)	1635 (236)	1297 (196)	1497 (234)
Citrate (mmol/24 hr)	3.60 (0.46)	2.31 (0.24)	2.45 (0.36)	3.86 (0.80)	1.99 (0.31)	3.13 (0.57)
Oxalate (mmol/24 hr)	0.25 (0.03)	0.26 (0.04)	0.23 (0.02)	0.29 (0.05)	0.29 (0.04)	0.27 (0.02)
Calcium (mmol/24 hr)	1.68 (0.24)	1.89 (0.31)	1.47 (0.20)	2.92 (0.47)	2.78 (0.53)	3.02 (0.39)
Magnesium (mmol/24 hr)	0.88 (0.17)	1.39 (0.35)	1.38 (0.23)	1.48 (0.28)	2.18 (0.52)	2.62 (0.72)
Sodium (mmol/24 hr)	172 (27)	165 (28)	133 (24)	166 (40)	179 (30)	144 (26)
Potassium (mmol/24 hr)	49.5 (7.4)	35.6 (4.1)	37.6 (7.5)	47.4 (12.3)	36.1 (5.8)	41.0 (9.3)
Urate (mmol/24 hr)	3.02 (0.42)	2.82 (0.34)	2.53 (0.28)	3.97 (0.77)	3.79 (0.45)	3.97 (0.53)
Creatinine (mmol/24 hr)	15.5 (1.1)	15.0 (1.2)	12.5 (0.9)	17.9 (1.2)	17.3 (1.1)	16.4 (1.7)
Phosphate (mmol/24 hr)	24.1 (0.86)	24.2 (3.5)	20.4 (2.7)	35.3 (6.16)	32.9 (2.6)	32.6 (2.9)
Chloride (mmol/24 hr)	157 (17.6)	142 (20)	133 (20)	126 (18)	162 (28)	129 (17)
Specific gravity ($g.cm^{-3}$)	1.01 (0.1)	1.01 (0.1)	1.01 (0.1)	1.01 (0.1)	1.01 (0.1)	1.01 (0.1)
RS CaOx	1.60 (0.34)	2.92 (0.90)	2.46 (0.60)	3.28 (0.76)	3.92 (0.68)	4.04 (0.81)
RS Brushite (exp-09)	0.24 (0.10)	0.61 (0.27)	0.52 (0.17)	0.75 (0.20)	1.13 (0.23)	1.05 (0.32)
RS Uric acid	1.59 (0.54)	1.50 (0.33)	1.20 (0.31)	1.93 (0.56)	1.98 (0.42)	1.91 (0.51)
Tiselius risk index	150 (26)	144 (22)	164 (26)	213 (29)	163 (23)	225 (23)

There were no significant changes in any of the variables upon consumption of *Folium pyrrosiae* in either race group (*day 0 vs day 7*). However, as was detected in the placebo protocol, urinary calcium and urinary phosphate was found to be significantly higher ($p<0.05$) in white subjects than in blacks on day 14 of the protocol.

Table 5.2b: Mean urinary parameters (SE) in black and white subjects before and after supplementation with *Folium pyrrosiae* and washout

Variables	**BLACKS (n=9)**			**WHITES (n=9)**		
	Baseline	**Post-treatment**	**Washout**	**Baseline**	**Post-treatment**	**Washout**
	day 0	**day 7**	**day 14**	**day 0**	**day 7**	**day 14**
pH	5.77 (0.15)	6.03 (0.16)	5.98 (0.21)	6.06 (1.04)	6.04 (0.17)	6.08 (0.11)
Volume (mL/24 hr)	1371 (90.7)	1298 (230)	1573 (158)	1430 (165)	1251 (137)	1635 (236)
Citrate (mmol/24 hr)	2.48 (1.06)	2.28 (0.29)	3.60 (0.46)	2.51 (0.34)	2.00 (0.28)	3.86 (0.80)
Oxalate (mmol/24 hr)	0.25 (0.02)	0.21 (0.02)	0.25 (0.03)	0.30 (0.03)	0.26 (0.03)	0.30 (0.05)
Calcium (mmol/24 hr)	1.77 (0.31)	1.55 (0.20)	1.68 (0.24)	2.32 (0.43)	2.45 (0.32)	2.92 (0.47)
Magnesium (mmol/24 hr)	1.20 (0.22)	1.71 (0.42)	0.88 (0.17)	2.31 (0.46)	2.07 (0.33)	1.48 (0.28)
Sodium (mmol/24 hr)	186 (24)	179 (23)	172 (27)	155 (17)	141 (17)	166 (40)
Potassium (mmol/24 hr)	50.2 (7.3)	38.7 (6.9)	49.5 (7.4)	40.3 (4.1)	38.1 (3.8)	47.4 (12.3)
Urate (mmol/24 hr)	2.50 (0.42)	3.04 (0.30)	3.02 (0.42)	3.82 (0.28)	3.80 (0.39)	3.97 (0.77)
Creatinine (mmol/24 hr)	13.9 (1.8)	14.3 (0.9)	15.5 (1.0)	16.4 (1.1)	17.2 (1.2)	17.9 (1.2)
Phosphate (mmol/24 hr)	25.3 (3.4)	22.6 (1.6)	24.1 (0.86)	29.0 (3.1)	29.8 (1.8)	35.3 (6.2)
Chloride (mmol/24 hr)	157 (17)	146 (17)	157 (17.6)	162 (16)	121 (10)	126 (18)
Specific gravity ($g.cm^{-3}$)	1.02 (0.1)	1.01 (0.1)	1.01 (0.1)	1.01 (0)	1.01 (0.1)	1.01 (0)
RS CaOx	2.36 (0.57)	2.92 (0.90)	1.60 (0.34)	3.45 (0.62)	4.10 (0.68)	3.28 (0.76)
RS Brushite	0.29 (0.09)	0.61 (0.27)	0.24 (0.10)	0.83 (0.27)	1.07 (0.28)	0.75 (0.20)
RS Uric acid	2.70 (0.91)	1.50 (0.33)	1.59 (0.54)	2.01 (0.57)	2.55 (0.88)	1.93 (0.50)
Tiselius risk index	165 (26)	136 (27)	150 (25)	183 (28)	165 (21)	173 (21)

5.3.3 Crystallization parameters

MSL, PSD, CaOx crystal nucleation and aggregation

Results of the urinary CaOx crystallization parameters are presented in Tables 5.3a,b and Figures 5.1a,b.

There was no significant change in any of the crystallization parameters post supplementation in both protocols. However, upon intergroup comparisons of baseline and washout data between the groups, the following differences were noted. In black subjects, MSL values were higher while the average CaOx PSD and aggregation inhibition were lower than in white subjects.

Table 5.3 a: Mean urinary parameters (SE) in black and white subjects before and after supplementation with starch placebo and post protocol

Variables	BLACKS (n=9)			WHITES (n=9)		
	Baseline **day 0**	***Post-treatment*** **day 7**	***Washout*** **day 14**	***Baseline*** **day 0**	***Post-treatment*** **day 7**	***Washout*** **day 14**
MSL (µmol/L)	78.3 (9.9)	85.0 (12.5)	88.3 (9.5)	40.0 (5.6)	33.3 (7.0)	61.7 (10.4)
PSD (µm)	8.38 (1.28)	6.78 (1.34)	5.83 (0.43)	10.6 (1.6)	10.9 (1.0)	9.55 (0.69)
% Inhibition of nucleation	-102 (16)	-256 (45)	-167 (44)	-122 (19)	-236 (15)	-140 (19.5)
% Inhibition of aggregation	-178 (74)	-103 (35)	-78.2 (73.4)	-84 (43)	-85 (26)	10.9 (28.8)

Table 5.3 b: Mean crystallization parameters (SE) in black and white subjects before and after supplementation with *Folium pyrrosiae* and post protocol

Variables	BLACKS (n=9)			WHITES (n=9)		
	Baseline **day 0**	***Post-treatment*** **day 7**	***Washout*** **day 14**	***Baseline*** **day 0**	***Post-treatment*** **day 7**	***Washout*** **day 14**
MSL (µmol/L)	96.7 (12.8)	101.7 (15.8)	76.7 (10.1)	56.7 (7.8)	61.7 (16.7)	40.0 (5.6)
PSD (µm)	5.83 (0.43)	7.19 (1.10)	8.80 (1.11)	9.55 (0.69)	8.32 (1.33)	8.38 (0.84)
% Inhibition of nucleation	-122 (52)	-72.6 (15.0)	-102 (16)	-173 (69)	-46.6 (13.6)	-122 (19)
% Inhibition of aggregation	-24.9 (29.9)	288 (393)	-178 (74)	27.3 (40.7)	10.9 (28.8)	-84 (43)

[^{14}C]-oxalate deposition experiments

The average precipitated [^{14}C]-oxalate in the urine of black (n=5) and white (n=5) subjects as a function of time is presented in Figures 5.1a and b, respectively. Experiments were performed pre- and post-treatment for both protocols (days 0 and 7). Precipitated [^{14}C]-oxalate at each time point of the 2 hour incubation period was determined in duplicate by a scintillation counter. Hence each data point presented in the respective graphs represents the average of 10 measurements.

There are three parameters of interest which can be derived from these plots:

- Gradient of the linear section which equals the rate of crystallization,
- Total percentage precipitation, and
- Time for crystallization to reach completion.

There were no statistically significant differences in the parameters of interest between the protocols in each group. However, intergroup comparisons (Figure 5.1a and b) revealed that in the urine of black subjects, precipitation continues for the entire duration of the experiment (120 minutes). While in white subjects it reaches completion at about 90 minutes (plateau in graphs), thereby suggesting that inhibition of crystallization occurs to a great extent in the former group.

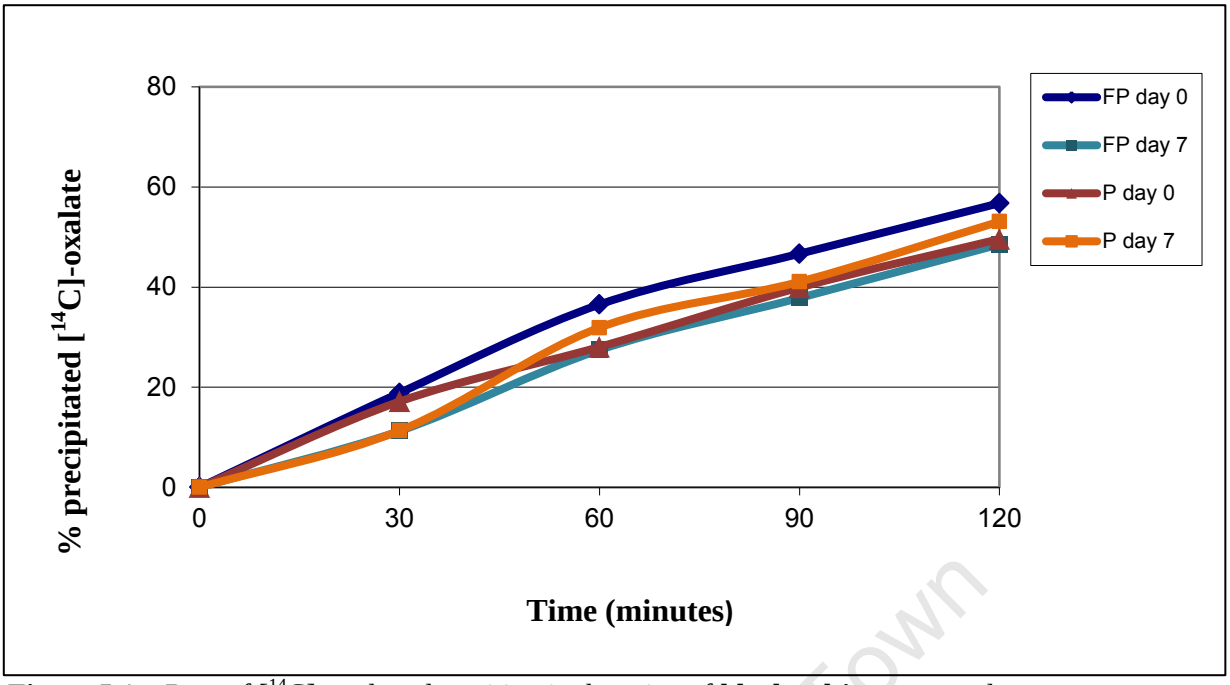


Figure 5.1a: Rate of [^{14}C]-oxalate deposition in the urine of **black subjects** pre-and post-treatment with *Folium pyrrosiae* **(FP)** and placebo **(P)**

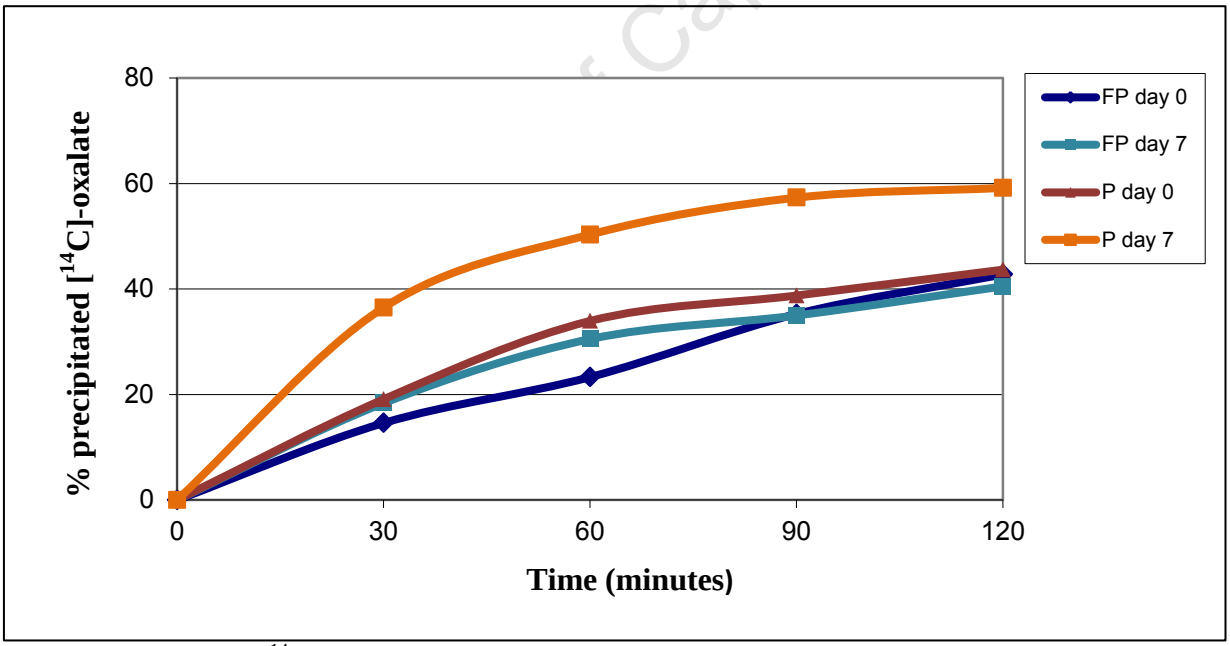


Figure 5.1b: Rate of [^{14}C]-oxalate deposition in the urine of **white subjects** pre-and post-treatment *Folium pyrrosiae* **(FP)** and placebo **(P)**

5.4 Discussion

Clinical trials on Chinese Medicines are generally difficult to conduct in China due to a lack of willing participants. Traditional methods of preparation by decoction (i.e. boiling plant material in water) have proven unpopular as most medicines taste bitter and it is impossible to standardise the technique of preparation (Zhao 2005). Therefore administering the plant extract in the form of a commercially produced capsule, the approach adopted in the present study, is a superior and more convenient technique for an *in vivo* study. Furthermore it facilitated the blinded-effect of the study as the plant extract was brown in colour and starch was white.

The influence of diet on urine chemistry is well-known and was discussed in detailed in chapter 1.1.4. Hence this investigation commenced with an inter-racial dietary analysis by means of evaluation of food questionnaires. Previous analysis of the typical diet of black and white subjects showed that whites have a significantly higher intake of total protein, vitamin C, vitamin B_6, fat, added sugar, calcium and magnesium whereas blacks have a higher intake of dietary oxalate (Lewandowski *et al.* 2005). However none the afore-mentioned dietary parameters or any others were significantly different between the groups in the present study thereby eliminating the possibility of any confounding dietary factors in the two cohorts of subjects used.

There were no significant changes in any urinary parameters in the placebo trial either. Starch served this purpose well as it was readily available, non-toxic and caused no interference to the variables of interest in this trial. There were also no significant changes in the relative supersaturations of the various stone-forming salts and TRI post treatment in either protocol. In a previous study using early morning urines derived from people of Chinese origin, significant changes in urinary parameters were also not observed (Ching 2007).

Although there were some fluctuations in urinary parameters following supplementation with *Folium pyrrosiae* (*pH, volume, citrate, oxalate, calcium, magnesium, sodium, potassium, urate, creatinine, phosphate and chloride*) none of these changes were statistically significant in either race group. However, attention is drawn to the decrease in oxalate concentration (Table 5.2 a) from 0.25 ± 0.03 to 0.21 ± 0.04 mmol/24 hr in blacks and from 0.30 ± 0.05 to 0.26 ± 0.04 mmol/24 hr in whites which is noteworthy due to its potential implications.

Despite there being no significant changes in urinary parameters post supplementation in this trial, calcium and phosphate was found to be statistically higher ($p<0.05$) in white males at baseline (day 0) and after the washout period (day 14) days in agreement with previously published urinary data (Lewandowski *et al*. 2001).

Folium pyrrosiae is purported to induce diuresis (Zhao 2005, Gohel *et al*. 2006). Specific gravity, which was determined in this study, is a measure of osmolality (i.e. the concentration of solute in solution) which is an indicator of the hydration state of the body. A superior method of evaluating the diuretic effect is by comparison of the change in the total 24 hour urinary volume. Neither of these parameters changed significantly after ingestion of *Folium pyrrosiae* indicating the absence of a diuretic effect.

Folium pyrrosiae demonstrated no effect on CaOx crystallization i.e. on MSL, PSD, crystal nucleation and aggregation. However comparison of these factors at baseline showed significant differences between the two race groups. The MSL of urine from blacks subjects was consistently higher ($p<0.05$) than that of white subjects (also reported for the *in vitro* studies in Chapter 3). The average CaOx PSD, which is an indication of crystal growth, was found to be greater in the urine of white subjects than in black subjects. These results correlate with the lower risk of stone formation in the black population group. However, the counter-intuitive finding here was the greater ($p<0.05$) inhibition of aggregation (which is associated with lower risk) in the urine of white subjects. [^{14}C]-oxalate deposition experiments demonstrated no significant differences in the rates of aggregation between the two race groups.

Traditional medicinal preparations and Western medicine have different principles of pathology and diagnosis. In Western medicine, a disease is viewed as an outcome of a pathogenic factor. However in traditional healing, a disease is diagnosed based on a multi-component system, i.e. an imbalance in the body and an outcome of a pathogenic factor (Lu *et al*. 1994, Mok 2006). A disease is referred to as a 'pattern of maladjustment' (*zheng* in Chinese, which can also be translated as syndrome). There are 4 such diagnostic states (1) ***heat*** which is characterised by fever, (2) an intolerance of ***cold*** in the body, (3) an ***excess*** accumulation of metabolic waste, and (4) a ***deficiency*** of nutrients; and there are 5 locations where these maladjustments can occur namely kidney, liver, heart, spleen and lung (Jiang 2005). Medicines are prescribed to assist the body to return to a 'normal' state by treating the

imbalance at the target organ. Traditional herbs have unique synergistic and sometimes antagonistic interactions with other constituent substances, with Western medicine and even some foods (Koide *et al*. 1995, Ikegami *et al*. 2003, Ikegami *et al.* 2004). A trained practitioner is able to administer the herbal formula with the correct balance.

Clearly, the system used in traditional medicinal systems for the evaluation of herbal preparations is very different to that used and accepted by Western practitioners as it lacks a sound scientific approach. Further evidence in support of this statement was revealed in a recent *in vivo* study involving Cystone® where no short (6 weeks) or long term effects (52 weeks) of the plant extract on urine chemistry of stone formers was detected (Erickson *et al*. 2011). In another recent *in vivo* study investigating the effect of Cystone® of cystine kidney stone patients, no favourable inhibitory effect was reported on stone burden either (Erickson *et al*. 2011a).

The purpose of this study was to provide greatly needed scientific backing on the pharmacodynamics of this widely used Chinese herbal preparation. Results of this study do not support the previously mooted suggestion that *Folium pyrrosiae* might be a therapeutic agent in the management of urolithiasis as no significant effects were detected on urine chemistry, MSL, RS, TRI, PSD, CaOx deposition and inhibition of nucleation and aggregation. However, urine from these subjects could be useful in future experiments in determining whether concentrations of herbal extracts used in the earlier *in vitro* experiments resemble those likely to be achieved *in vivo*.

Isolation, identification and structural analysis of potentially bioactive compounds in *Folium pyrrosiae*

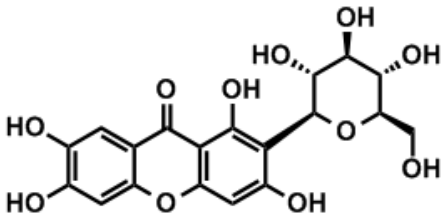
6.1 Introduction

In the previous chapters of this thesis, the inhibitory activity of *Folium pyrrosiae* on CaOx risk factors (crystallization and crystal-cell binding) in *in vitro* and *in vivo* experiments has been described. It was shown that this herb decreased CaOx crystal growth (as determined by SEM and MSMPR experiments, Table 3.8) and inhibited crystal-cell binding when used to either pre-coat crystals or cells, or when present in the binding medium (chapter 4). Once the inhibitory effects of an agent have been established, it is of interest to identify and determine the structure of its constituent bioactive compounds to determine the mechanisms by which the compounds act. The present chapter describes the elucidation of the structures isolated from *Folium pyrrosiae*.

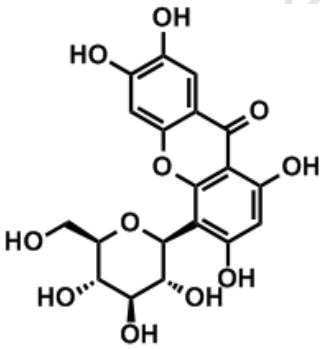
In an analytical study carried out by Li and Tong (1992) on *Folium pyrrosiae*, 3 well-known phytochemicals were identified. These workers employed high-performance liquid chromatography to analyse a methanol extract of the dried plant material and reported the presence of *mangiferin* and *isomangiferin* (two xanthone derivatives) as well as *chlorogenic acid* (a polyphenol). In fact, the study included compositional analysis of 7 species of this particular genus which was isolated from 17 districts in China. The 3 aforementioned constituents were detected in varying concentrations between species and in the same species obtained from the different districts (Li and Tong 1992). The structures of these compounds are presented in Figure 6.1.

Mangiferin (*1,3,6,7-tetrahydroxy-2-[3,4,5-trihydroxy-6-(hydroxymethyl)oxan-2-yl]xanthen-9-one*) has well-known immunotherapeutic effects. It has demonstrated strong anti-oxidant, anti-lipid peroxidation and anti-diabetic activities. In addition, it can promote wound healing and may play a role in preventing cancer, autoimmune disorders, atherosclerosis and coronary heart disease (Zheng and Lu 1990, Yoshimi *et al*. 2001, Leiro *et al*. 2003, Muruganandan *et al*. 2005, Shah *et al*. 2010). **Isomangiferin** (*1,3,6,7-tetrahydroxy-4-[3,4,5-trihydroxy-6-*

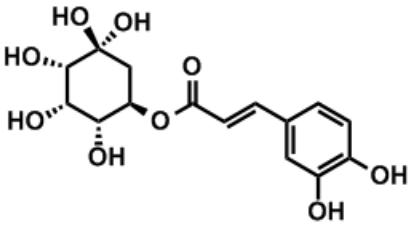
(hydroxymethyl)oxan-2-yl]xanthen-9-one), an isomer of mangiferin, is known to act as an antiviral (Zheng and Lu 1990). **Chlorogenic acid** (*3-[(E)-3-(3,4-dihydroxyphenyl)prop-2-enoyl]oxy-1,4,5-trihydroxy-cyclohexane-1-carboxylic acid*) has reported anti-oxidant and anti-carcinogenic properties, and plays a role in the homeostatic regulation of blood glucose (Hemmerle *et al*. 1997, Gonthier *et al*. 2003).



(a) *1,3,6,7-tetrahydroxy-2-[3,4,5-trihydroxy-6-(hydroxymethyl)oxan-2-yl]xanthen-9-one*



(b) *1,3,6,7-tetrahydroxy-4-[3,4,5-trihydroxy-6-(hydroxymethyl)oxan-2-yl]xanthen-9-one*



(c) *3-[(E)-3-(3,4-dihydroxyphenyl)prop-2-enoyl]oxy-1,4,5-trihydroxy-cyclohexane-1-carboxylic acid*

Figure 6.1: Structures of mangiferin **(a)**, isomangiferin **(b)** and chlorogenic acid **(c)**

This chapter describes an attempt to identify and characterize other molecules in *Folium pyrrosiae* and establish a protocol for characterization of other such herbal preparations.

6.2 Methods

In order to characterize phytochemicals in *Folium pyrrosiae*, these compounds were isolated from the dried plant material and purified prior to analysis. An aqueous extract of *Folium pyrrosiae* was subjected to sequential liquid-liquid extractions with organic solvents ethyl acetate (EtOAc) and butanol (BuOH). These extracts were subsequently purified by column-chromatography and thin-layer chromatography (TLC). Further analysis was performed by means of NMR spectroscopy and mass spectrometry (MS). This experimental procedure was designed in collaboration with Professor Thozamile Mabusela (*Herbal Science and Medicine Institute, University of the Western Cape, South Africa*). A schematic is presented in Figure 6.2 and further details are provided later in the chapter.

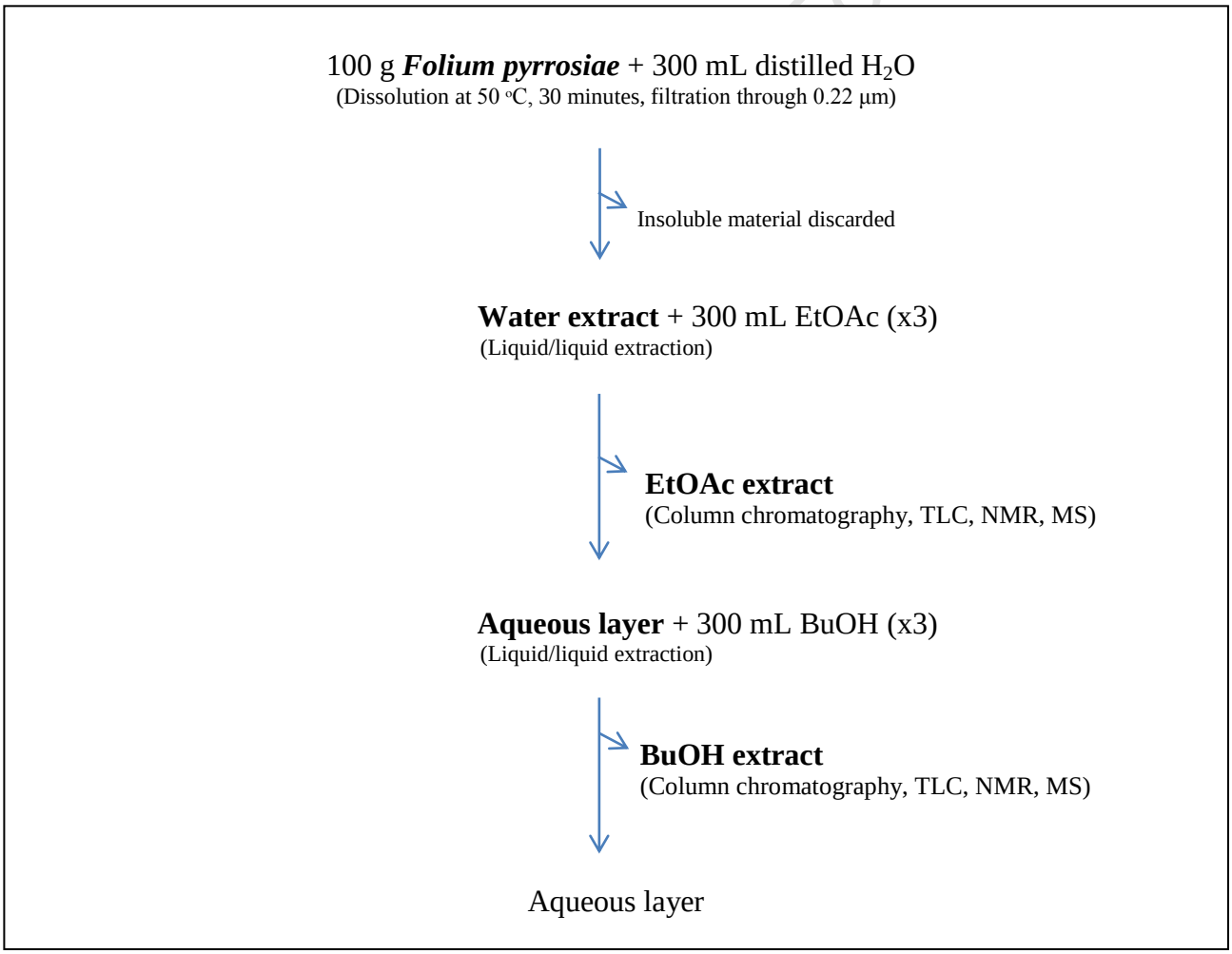


Figure 6.2: A flow diagram summarizing the isolation and purification of bioactive molecules in *Folium pyrrosiae*

Liquid-liquid extractions

Liquid-liquid extraction is a method that is used to separate compounds based on their relative solubilities into 2 immiscible liquids (Zubick 1992). *Folium pyrrosiae* (100 g) was weighed into a conical flask into which distilled water was added (300 mL). Gentle heating with stirring (*Labcon, Johannesburg, South Africa*) was applied for 30 minutes to facilitate dissolution. The solution was microfiltered (0.22 μm) to remove insoluble material. This water-soluble extract (~ 290 mL) was subjected to extraction by EtOAc (*Sigma-Aldrich, Cape Town, South Africa*) as described below.

EtOAc (300 mL) was added to the water-soluble extract of *Folium pyrrosiae* which was placed in a separating funnel. The separating funnel was secured at both ends (rubber stopper on the top and tap at the bottom) and the liquid mixture was shaken vigorously. During the first 2 minutes, the tap of the separating funnel was opened at 5 second intervals to release built-up pressure. The liquids were shaken for a period of 10 minutes in total. Thereafter the separating funnel was clamped to a retort stand and allowed to stand for 2 hours to enable separation of the aqueous and organic layers. The EtOAc (lower) layer was decanted and the extraction process was repeated twice, each time using a further 300 mL of EtOAc. The organic extracts were pooled and set aside for further analysis at a later stage and the aqueous extract remained in the separating funnel.

An aliquot of 300 mL BuOH (*Kimix, Cape Town, South Africa*) was added to the water extract of *Folium pyrrosiae* in the separating funnel. The extraction procedure was repeated, i.e. the separating funnel was secured at both ends, the liquid mixture was shaken vigorously and the pressure inside the flask was released intermittently. Shaking continued for 10 minutes after which the separating funnel was clamped to a retort stand and allowed to stand overnight to allow separation of the 2 layers. This longer time period (than the 2 hours above) was allowed due to the formation of an emulsion at the liquid-liquid interface; however, after a sufficient standing period undisturbed, a clear definition between the aqueous and organic layers was apparent. The lower organic layer was carefully decanted and the extraction process was repeated twice, each time using a further 300 mL of BuOH.

The organic extracts (EtOAc and BuOH) were analyzed individually. Anhydrous magnesium sulphate ($MgSO_4$) was used as a drying agent, i.e. to remove residual water in the organic extracts. Drying agent was added to the organic extracts until $MgSO_4$ granules were free-flowing, an indication that all moisture has been absorbed (Zubick 1992). $MgSO_4$ was incubated firstly in an oven at 100 ^oC for 1 hour to ensure that it was free of moisture as the anhydrous form is hygroscopic.

The water-free organic extracts were evaporated to dryness on a rotorary evaporator (*Rotovap A201, Switzerland*). At this stage, ~ 3 g of dried material was yielded in each case which was subjected to purification by column chromatography.

Column chromatography

Column chromatography is widely used in organic and inorganic chemistry to separate and purify compounds. This technique is based on the distribution of components in a mixture between a mobile phase (solvent or eluent) and an inert stationary phase (column packing) which arises as a result of differences in polarity. In order to prepare a column, the stationary phase is made into a slurry with the eluent and poured into the column and allowed to settle (Vogel 1989, Zubick 1992). Care is taken to ensure that no air bubbles are trapped in the stationary phase. Should such bubbles occur, then the side of the column is tapped gently to facilitate removal of these air bubbles.

The dried liquid extract to be separated is dissolved in 2 mL eluent and loaded onto the column. Thereafter more solvent is added to the column and compounds are separated as they travel down the column (or elute) at different rates based on their affinity for the mobile phase. At the bottom end of the column, the solvent is collected in portions called fractions (Vogel 1989, Zubick 1992).

Polar components adhere strongly to the stationary phase and elute much slower than less polar compounds. Ideally a mixture will separate into discrete bands and elution is continued until all compounds have been washed through the column. Generally, more than one solvent is needed

to move compounds in a mixture through a column. Therefore a gradient elution is performed where the polarity of the solvent is gradually increased (Vogel 1989, Zubick 1992).

A slower solvent flow-rate enables better separation. When compounds are coloured, they are easily identified; however, colourless compounds require the use of other techniques (e.g. TLC) for identification. After identification, the appropriate fractions are pooled and evaporated to dryness to yield a pure product.

In order to separate the EtOAc and BuOH extracts into their constituent compounds, columns were set up separately and the following conditions were applied:

Column dimensions: 70 x 1.5 cm

Stationary phase: 200 g Sephadex G-100 (*Sigma-Aldrich, South Africa*)

Eluents: hexane (*Kimix, Cape Town, South Africa*), toluene (*Kimix, Cape Town, South Africa*) and methanol (*Kimix, Cape Town, South Africa*)

Elution gradients:

- 100 mL aliquots of hexane : toluene, in the order (100:0, 90:10, 80:20, 70:30, 60:40, 50:50, 40:60, 30:70, 20:80, 10:90, 0:100)
- 100 mL aliquots of toluene : methanol, in the order (100:0, 90:10, 80:20, 70:30, 60:40, 50:50, 40:60, 30:70, 20:80, 10:90, 0:100)
- 100 mL water

These solvents were selected as they covered the spectrum of polarity, from non-polar (hexane) to polar (methanol). Fractions were collected in 10 mL aliquots using an automated fraction collector (*Bio-Rad 2110, Germany*). Each aliquot was analyzed by TLC in order to detect the presence of any organic compounds.

Thin-layer chromatography

TLC is a technique used to detect the presence of organic materials. It is based on the same principles of column chromatography, namely that compounds distribute themselves between the mobile and stationary phases due to differences in polarity. However, in the case of TLC, the solid phase (usually alumina or silica) is attached to a flat plate in a thin layer and the mobile phase carrying the compounds washes over the plate. These plates can be made of glass or metal (Vogel 1989, Zubick 1992). A schematic of a TLC plate is given in Figure 6.3.

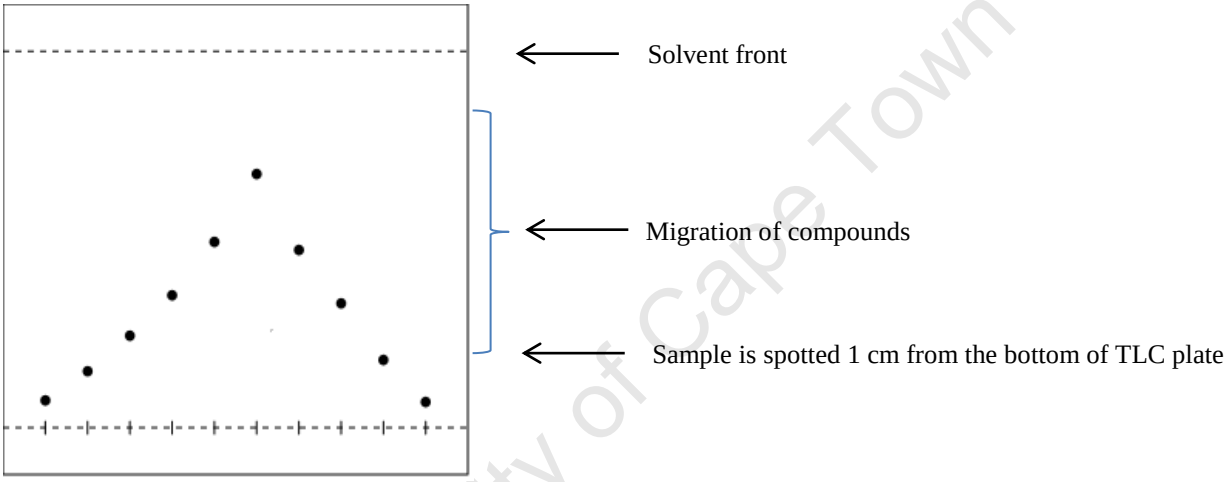


Figure 6.3: A schematic of a TLC plate showing the solvent front and migration of sample

When perfoming TLC, a small amount of sample is dissolved in an eluent. Using a caplillary dropper, the sample is loaded onto the plate 1 cm from the bottom and allowed to dry. This step is repeated a few times to ensure that sufficient amount of material is present for detection. Once the sample is loaded, the plate is placed in a covered glass container (a beaker and coverslip may be used) containing the eluent. Care is taken to ensure that the eluent level is below where the sample is loaded, i.e. less than 1 cm from the bottom of the plate. This precaution is taken to prevent sample from moving down the plate and washing into the eluent. The solvent will slowly rise up the plate and carry the componds at different rates. When the solvent front is approximately 1cm from the top of the plate, the plate is removed from the glass chamber and the position of the solvent is marked with a pencil. Thereafter the plate is viewed under ultra-violet light and the position of the spots (compounds) are marked (Vogel 1989, Zubick 1992).

Following column chromatography, all fractions collected (approximately 230 for each column) were analyzed by TLC (*Merck, South Africa, South Africa*). A combination of hexane, toluene and methanol at a ratio of 1:1:1 was used as the eluent. Post TLC analysis, plates were examined under ultra-violet light (ʎ = 254 nm, *Lamag Instruments*) and the presence of compounds was detected by the appearance of yellow/brown dots which migrated up the TLC plate. Furthermore, the distribution of the spots on the plates (as shown in Figure 6.3) indicates the start and completion of elution of a compound (Vogel 1989).

TLC data showed two distinct peaks in the EtOAc extract and five in the BuOH extract. The respective fractions were pooled and the solvents were evaporated. Sometimes further purification is needed. However due to the slow elution times that the columns were subjected to (which allows better separation) and that fractions were collected in small volumes (in aliquots of 10 mL), it was deemed unnecessary in this case. The separated compounds from the EtOAc were named ET1 and ET2, and those isolated from the BuOH extract were named BU1, BU2, BU3, BU4 and BU5 respectively. These supposedly pure compounds were analyzed by NMR spectroscopy and mass spectrometry for confirmation thereof.

Nuclear magnetic resonance spectroscopy

Nuclear magnetic resonance (NMR) was discovered in 1946 independently by Edward Purcell and Felix Bloch. It is a powerful and complex analytical technique used in elucidation of molecular structures of liquid and solid organic materials. A great advantage of this method is that it is non-destructive and samples can be recovered after analysis without any modification (Fox and Whitesell 1997, Laidler and Meiser 1999).

NMR analysis yields spectra of magnetic sensitive nuclei (e.g. ^{1}H and ^{13}C) whose spin depends on the surrounding structural environment. Proton (^{1}H) and carbon (^{13}C) spectra provide fingerprint data which can be used to determine the identity and purity of an unknown sample. Confirmation and unambiguous determination of molecular structures follows from the use of 2D NMR correlation spectra (Fox and Whitesell 1997, Laidler and Meiser 1999).

^{1}H NMR forms the starting point in structural analysis and requires the least amount of sample. The chemical shift of a proton is the frequency reported in parts per million (ppm) that is expressed relative to tetramethylsilane (TMS), a standard reference compound. Proton signals range between 0 and ~12 ppm. The chemical shift depends on the surrounding electronic environment and can be regarded as a distribution of protons according to their electron density. Peaks of a lower frequency are 'downfield' and arise from protons of low electron density, whereas peaks 'upfield' are due to electron-rich protons. The splitting of the peaks results from spin-spin coupling with neighbouring protons and under optimal conditions, the relative intensities of the signals may be correlated with the amount of nuclei producing these signals. However, in both ^{1}H NMR and ^{13}C NMR studies, the chemical shift values of compounds are affected significantly by changes in solvent, temperature and pH (Williams and Fleming 1995).

The **^{13}C NMR** spectra are often much simpler spectra than ^{1}H NMR and provide two basic pieces of information: (1) the number of distinct signals which correspond to the number of different types of carbon atoms in the structure, and (2) the chemical shifts of the ^{13}C NMR signals which are relatively characteristic and more amenable to assignment by inspection than those of protons (Fox and Whitesell 1997). **DEPT** (Distortionless Enhancement by Polarization Transfer) ^{13}C spectrum is a very useful method to differentiate between CH, CH_2, CH_3 and quaternary carbons.

The chemical shifts of ^{13}C are also recorded relative to TMS and carbon signals range between 0 and 250 ppm. There are three main distinguishable regions: (1) carboxyl and carbonyl groups (~170 ppm), (2) anomeric carbons (110-90 ppm), and (3) the remaining ring carbon atoms (80-65 ppm) and primary alcohols (65-60 ppm) (Jarrell *et al*. 1979, Williams and Fleming 1995).

In order to further resolve the ^{1}H and ^{13}C assignments, 2D NMR experiments are employed, such as proton-proton chemical shift correlation spectroscopy (**COSY**) and proton-carbon heteronuclear chemical shift correlation spectroscopy (HETCOR). The 2D pulse sequences are made up of single or multiple pulses and delays prior to the final observation of the signal. The output of 2D spectra is in the form of a contour plot with frequency axes. Both axes are

connected by scalar coupling (homo- or heteronuclear) and give chemical shift information (Williams and Fleming 1995).

COSY spectra consist of a complex diagonal with pairs of off-diagonal peaks which arise from each pair of scalar coupled protons. The coupling can be worked out starting from a single unambiguous assignment. Multiple relay COSY can be used to resolve ambiguities arising from proton spectra (Williams and Fleming 1995).

HETCOR maps contain ^{1}H chemical shifts along one axis and ^{13}C chemical shifts along the other axis. The peaks on the map arise due to connections between a ^{13}C nucleus and a proton. HETCOR can be used to investigate various types of connectivity, e.g. one-bond coupling, long-range couplings or relayed correlation (Williams and Fleming 1995). There are basically two types of experiments in this category: those that utilize multiple quantum transitions during the evolution time (e.g. **HMBC**) and those using single quantum transitions during the evolution time (e.g. **HSQC**). HSQC experiments correlate protons with their directly attached heteronuclei (Williams and Fleming 1995).

The purified compounds of the EtOAc and BuOH extracts were dissolved in acetone and NMR spectra, as described above, were recorded at 400 MHz at 30 ˚C. NMR spectra were processed using standard *Varian* software.

Mass spectrometry

MS is an analytical technique to characterize molecules based on the measurement of molecular mass. The three essential components that make up a Mass Spectrometer are the ion source, the mass analyzer and the detector. The ion source ionizes the sample usually by the loss of an electron to form a cation. The main function of the mass analyzer is to separate ions according to their mass-to-charge (m/z) ratio. The detector monitors the ion current and a signal is recorded in the form of a mass spectrum. The m/z values of the ions are plotted against their intensities to show the number of species present in the sample, the molecular mass of each component and the relative abundance of the different species. The fragmentation patterns shows peaks

corresponding to the loss of specific fragments in a molecule, hence imparting structural information (Siuzdak 1996, Cole 1997).

The purified compounds of the EtOAc extract was analyzed on an electrospray ionization mass spectrometer (*Waters API Q-TOF Ultima, Massachusetts, USA*) under the following operating conditions: capillary voltage – 3.5kV; source temperature – 120 ºC; desolvation temperature – 350 ºC; calibration – NaF; scan – 200-2000 m/z.

6.3 Results and discussion

Compounds ET1 and ET2 were white and yellow amorphous powdery material, respectively. Compounds BU1, BU2, BU3, BU4 and BU5 were brown, liquid-like substances. Following analysis by NMR, ET1 and ET2 provided decipherable spectra. However, all compounds isolated from the butanol extract had not maintained integrity during the purification process hence structure elucidation was not possible. All spectral data are presented in Appendix 5.

ET2 was isolated in greater abundance than ET1 therefore identification of this compound was given priority. Structural elucidation of a natural product is undoubted a challenging task. Various experiments were performed and data were analyzed concurrently. Based on these findings and consultation with previously published data, the structure of ET2 was proposed to be ***5-(3-(5,5-dihydroxy-3-oxopentyl)phenoxy)-2-hydroxy-5H-indene-6-carboxylic acid***. The structure of this compound is presented in Figure 6.4 and evidence in support thereof is presented below.

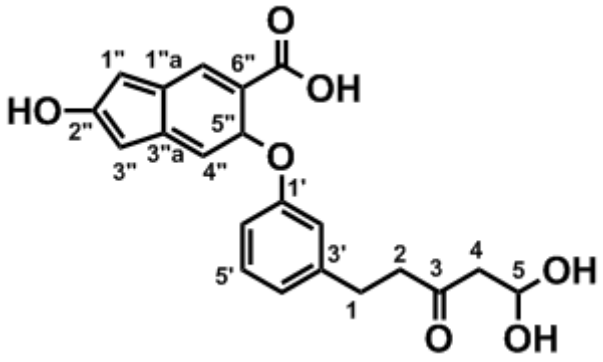


Figure 6.4: The structure of *5-(3-(5,5-dihydroxy-3-oxopentyl)phenoxy)-2-hydroxy-5H-indene-6-carboxylic acid*

Table 6.1: NMR assignments of *5-(3-(5,5-dihydroxy-3-oxopentyl)phenoxy)-2-hydroxy-5H-indene-6-carboxylic acid*

Assignments	**δ_C** (ppm)	**δ_H** (ppm)	**Comments**
1	40.1	3.33	DEPT indicates CH_2; COSY to H at 3.04 ppm.
2	31.1	3.04	DEPT indicates CH_2; COSY to H at 3.33 ppm.
3	195.4	-	Assignment based on literature values[1] of a C=O; HMBC shows correlation to H's at 2.85 and 3.04 ppm.
4	43.8	2.85; 3.16	DEPT indicates CH_2; HSQC confirms attachment of both protons to the respective carbon; COSY to H 5.59 ppm.
5	80.1	5.59; OH – 12.14	DEPT indicates CH; COSY to H's at 2.85 and 5.59 ppm. OH assignment was based on literature values[1,2].
1'	139.2	-	Quaternary carbon.
2'	127.4	7.58	Aromatic carbon and proton assignments were based on DEPT and HSQC results as well as literature comparisons for signals in this chemical shift range[1,3,4]. Cross peaks also detected in COSY.
3'	141.2	-	
4'	127.4		
5'	129.5	7.45	
6'	127.0	7.19	

1''	97.1	5.98	DEPT indicates CH; HMBC shows correlations to H at 6.02 ppm; COSY indicates no H's on adjacent carbons hence double bond proposed between *1''* and *1''a*.
1''a	164.4	-	DEPT indicates quaternary C; HMBC shows coupling to H's at *1''*, *3''*, *5''* and *4''*.
2''	166.3	OH – 9.55	Assignment based on literature value[5] of a C-OH in a 5 membered carbon cyclic motif.
3''	96.0	6.02	DEPT indicates CH; HMBC shows correlations to H at 5.98 ppm; COSY indicates no H's on adjacent carbons; *1''* and *3''* are proposed to be opposite each other.
3''a	163.1	-	DEPT indicates quaternary C; HMBC shows coupling to H's at *3''*, *4''* and *5''*.
4''	108.9	6.43	DEPT indicates CH; COSY shows correlation to H at 7.8 ppm and no other protons hence double bond proposed between *3''a* and *4''*.
5''	133.8	7.85	DEPT indicates CH; COSY shows correlation to H at 6.43; long range coupling to *1''a* and *3''a* shown in HMBC
6''	112.0	-	DEPT indicates quaternary C hence the double bond proposed between 6'' and 7''; long range coupling to H's at *4''* and *7''* ppm shown in HMBC
7''	103.7	6.34	DEPT indicates CH; HMBC shows a small coupling to *1''a* and *3''a*.
COOH	170.0	OH – 13.50	Assignment based on literature values of a COOH[1,6,7].

[1]Nagem *et al*. 1997; [2]Corse and Lundin 1970; [3]Sun *et al*. 2006; [4]Chen *et al*. 2007; [5]Schweiger *et al*. 2010; [6]Berrigi *et al*. 2003; [7]Kim *et al*. 2006

δ_C (ppm) – chemical shift of C atom in spectrum

δ_H (ppm) – chemical shift of H atom in spectrum

Further extensive structural confirmation can be achieved by running the ^{1}H NMR spectrum in deuterium oxide (D_2O). This solvent freely interchanges the hydroxyl protons hence all OH signals will disappear from the ^{1}H spectrum thereby confirming their presence. In addition to this, methylation and acetylation of the compound (to the carboxyl and hydroxyl groups) may be performed to obtain further structural validation (Faizi *et al*. 2006).

The melting point range of a compound (a single small range) is an indicator of its purity. The melting point of ET2, determined using a hot stage microscope (*Reichert-Jung Thermovar, Germany*), was found to be 56-59 ^{o}C.

The molecular weight of the proposed structure of ET2 (Figure 6.4) is 384 g.mol^{-1} and its elemental composition is C = 65.6%, H = 5.2% and O = 29.1%. Microanalysis of ET2 (*service provided by the Department of Chemistry, University of Cape Town*) provided the following elemental composition: C = 66%, H = 5% and O = 29%, which is in close agreement with that of the proposed structure of ET2.

The fragmentation pattern in the MS of ET2 is presented in Figure 6.5. The major peak at 352 g.mol^{-1} is likely due to the loss of the hydroxyl groups on C*5* (indicated by the blue bar). The second most abundant peak at 257 g.mol^{-1} is attributed to fragmentation (indicated by the red bar) via the McLafferty rearrangement reaction (McLafferty 1959) which can occur in a molecule containing a keto-group that undergoes β-cleavage accompanied by the gain of the γ-hydrogen atom.

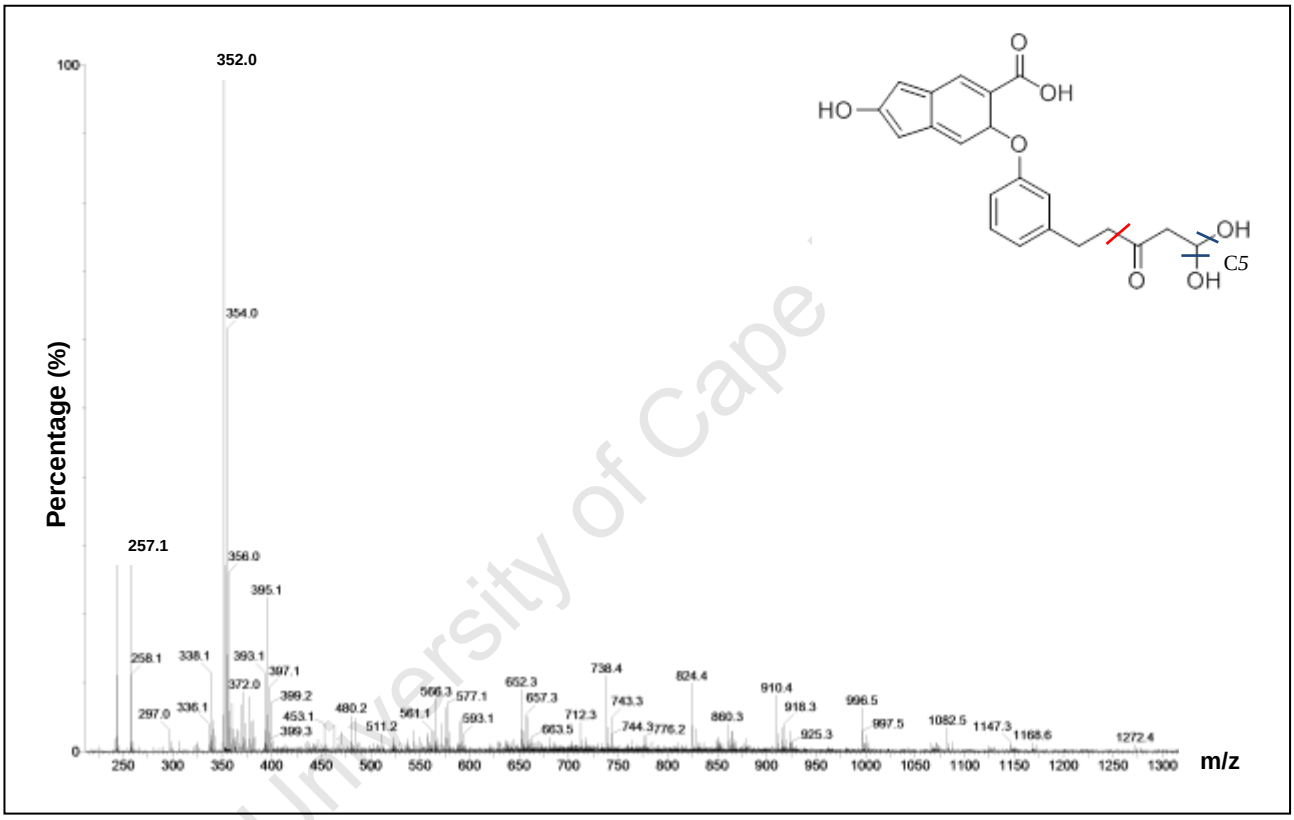


Figure 6.5: The MS fragmentation pattern of ET2

6.4 Conclusion

The protocol implemented in the isolation of compounds from *Folium pyrrosiae* proved to be successful in obtaining two pure compounds from the EtOAc extract (ET1 and ET2). ET2 was extracted in abundance compared to ET1 therefore the challenging task of structural elucidation of this compound was attempted.

The experimental data presented in this chapter strongly supports the structure of ET2 to be *5-(3-(5,5-dihydroxy-3-oxopentyl)phenoxy)-2-hydroxy-5H-indene-6-carboxylic acid*. This phytochemical is believed to be a novel compound as there has been no characterization studies reported in literature previously. Further investigations of its bioactive properties are highly warranted in order to determine if it has similar biological activity to the other compounds identified in *Folium pyrrosiae* that were mentioned earlier.

Concluding remarks

In concluding this thesis, it is appropriate to assess the extent to which the objectives have been achieved. This project was based on the well-established observation that stone patients are very likely to have recurrent episodes, and despite years of research, there is still no ideal drug to cure the disease. Patients are usually administered with the same contemporary Western medicines over long periods of time and unpleasant side-effects have been reported. In recent years, herbal remedies have emerged to be useful with fewer known side-effects and are therefore being considered suitable for long-term treatment.

The primary focus of this project was directed towards developing and establishing models for *in vitro* and *in vivo* investigations of herbal remedies purported to be effective in the treatment of urolithiasis. Six such preparations (*Folium pyrrosiae*, *Desmodium styracifolium*, *Hylocerus trigonus*, *Phyllanthus niruri*, *Orthosiphon stamineus* and Cystone®) were utilized in this development process. The secondary objective was to investigate the renal response of subjects from South Africa's black and white population groups to the administration of one of these remedies (*Folium pyrrosiae*), with a view to gaining insights into why the stone incidence in the former group is extremely rare.

Intensive literature reviews have revealed two major mechanisms by which stone formation may be inhibited, namely by inhibition of the crystallization processes of stone-forming salts (free-particle theory) and inhibition of crystal-cell adhesion (free-particle theory).

The *in vitro* effects of the plant extracts on crystallization processes (i.e. nucleation, growth and aggregation) are reported. All plant extracts inhibited one or more of these processes in SU. These favourable results gave impetus for further *in vitro* studies in real urine collected from healthy South African black and white males. However, fewer statistically significant effects on crystallization were observed in real urine. Since human urine contains urinary proteins and other macromolecules which SU does not, this finding drew attention to the need to introduce physiological conditions into the study.

Different urinary responses were induced by the plant extracts in the two population groups, suggesting that renal handling differences exist between the races. This confirms previous literature reports.

The most noteworthy result from the *in vitro* study was the ability of the plant extracts to favour the crystallization of COD over COM since the former has a lesser binding affinity for renal epithelial cells. This outcome prompted the exploration of another facet of urolithiasis research, namely crystal-cell binding which has been identified as a critical step in the genesis of a kidney stone.

A basic crystal-cell binding assay was performed. Various modifications were introduced to investigate possible mechanisms by which the plant extracts operate and to mimic physiological conditions. The potency of the six plant extracts under investigation was demonstrated by their ability to significantly decrease binding of CaOx crystals (inorganic and urinary) to renal epithelial cells in both aqueous media and real urine.

Despite all the promising results mentioned earlier, *Folium pyrrosiae* demonstrated no effect on urine chemistry and crystallization properties *in vivo*. Hence no scientific support for its use as a therapeutic agent for kidney stone disease. This finding also demonstrates that the system used to evaluate herbal remedies in traditional medicinal systems is different to that used and accepted by Western practitioners and lacks a sound scientific approach. As mentioned previously, the diagnosis (and subsequent treatment) of diseases in the traditional medicinal system significantly varies to that of modern day medicine.

In the present project, a successful protocol was established to purify and characterize potentially bioactive molecules. This was implemented in the analysis of *Folium pyrrosiae*. A novel compound, *5-(3-(5,5-dihydroxy-3-oxopentyl)phenoxy)-2-hydroxy-5H-indene-6-carboxylic acid*, was identified. It would be of great interest to determine the immunological activity of this molecule.

With respect to understanding the rarity of stone incidence in black South Africans, this thesis provided no new information but confirmed previous findings which were mentioned above. In crystal-cell binding assays, urinary crystals from black subjects showed reduced binding compared

to those of white subjects. Qualitative analysis of urinary proteins contained in these crystals from the two race groups would be another avenue of interest in future studies.

The key contribution of this thesis to the pool of knowledge about kidney stone disease has been to demonstrate that herbal preparations have the potential for becoming therapeutic and prophylactic agents in the treatment and management of this condition. Further scientific and clinical investigations are needed before such regimens are implemented. Ultimately, the efficacy question can probably only be answered with randomised placebo-controlled studies in patients, using stone formation as the end-point.

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Appendix 1

1.1) MSL of SU and SU+Shi Wei and SU+JQC (in triplicate)

no. of particles reported is the average of 2 readings taken

	No. of particles								
[Na2Ox]	*SU 1*	*SU 2*	*SU 3*	*SU+SW 1*	*SU+SW 2*	*SU+SW 3*	*SU+JQC 1*	*SU+JQC 2*	*SU+JQC 3*
0	2080	1714	1698	2547	2547	1195	2175	3510	112
15				3796	2233		1846	3626	
30				1924	2204		1638	2325	
45	2048	1047	2768	3049	3374	3232	3095	2697	3773
60	2073	1067	1958	3255	1985	3790	2129	3303	5534
75	2500	1287	2632	5194	4326	5846	4211	3152	5311
90	2845	1468	2071	4092	1391	6321	6638	3770	3953
105	2085	1095	9742	4983	7722	6171	9865	5859	3014
120	22071	11096	7322	15783	23104	12709	6441	12869	7618
135	25078	12607	8625	29380	21532	14829	20637	5731	4759
150	28967	14559	32276	27014	41334	40439	31012	36656	23617
165	43907	22036	9824	33663	38452	36229	24112	32647	19935
180				42562	43392	38653	14699	42105	25308
195				43177	43049		44267	44973	32975

1.2) MSL of SU and S

no. of particles reported is th

	No. of parti
[Na2Ox]	*SU 1*
15	1398
30	2380
45	3089
60	3865
75	3645
90	3759
105	3732
120	11926
135	23613
150	19553
165	

	AU 1	AU 2	AU 3	PN 1	PN 2	PN 3	JT 1	JT 2	JT 3	CYS 1	CYS 2	CYS 3
0	362	367	360	927	1093	700	420	454	356	1211	1782	2551
30	1002	1351	1785	1129	1494	1056	1366	837	589	1411	2121	2678
45	1464	3382	1807	2053	945	2027	494	238	1151	1511	2273	2230
60	811	1556	478	2197	892	1884	2051	610	1226	2566	794	2791
75	2173	2529	897	2774	1325	2995	547	736	480	2260	938	4053
90	22774	5125	3091	5526	5602	5515	789	465	1559	2571	1431	3573
105	19982	29906	5235	6344	6656	6521	4660	1948	2994	4782	6360	8744
120	27030		11980			6006	10749	13749	11559	14334	18191	22874
135						17926						
150												

U+Odyvato (in triplicate)

e average of 2 readings taken

cles				
SU 2	*SU 3*	*SU+ODY 1*	*SU+ODY 2*	*SU+ODY 3*
				1402
2290	2335	2456	1543	2188
4135	3612	1556	2324	2053
2893	3379	800	4310	1248
3811	3728	4996	2383	1525
3700	3729	1652	4989	2348
16355	10043	5060	7364	2506
14950	13438	8381	3378	5102
43256	33434	6837	10276	35974
	19777	7904	11676	36724
		30695	18431	35080

1.4) MSL of SU, SU+PN, SU+OS and SU+CYS (in triplicate)

no. of particles reported is the average of 2 readings taken

	No. of particles								
[Na2Ox]	*SU 1*	*SU 2*	*SU 3*	*SU+PN 1*	*SU+PN 2*	*SU+PN 3*	*SU+OS 1*	*SU+OS 2*	*SU+OS 3*
0	362	367	360	1093	700	925	420	454	356
30	1002	1351	1785	1494			1366	837	
45	1464	3382	1807	945	2027	1332	494	238	1151
60	811	1556	478	892	1884	1296	2051	610	1226
75	2173	2529	897	1325	2995	1554	547	736	480
90	18889	5125	3091	5602	5515	1066	789	465	1559
105	19982	29906	5235	6656	6521	4080	4660	1948	2994
120	27030		11980		6006	17434	10749	13749	11559
135					17926				

SU+CYS 1	SU+CYS 2	SU+CYS 3
1211	1782	2551
1411	2121	2678
1511	2273	2230
2566	794	2791
2260	938	4053
2571	1431	3573
4782	6360	8744
14334	18191	22874

white urine

							PROTEIN	
blank		0.000	0.000	0.000	0.000		60.853	
		0.001	0.001	0.002	0.001	average	324.313	
		0.003	0.004	0.004	0.004	0.002	109.536	
						minus blank	**ug/ml**	
control		0.284	0.285	0.285	0.285	0.283	2.715338	
		0.391	0.394	0.399	0.395	0.393	3.772014	
		0.292	0.293	0.295	0.293	0.291	2.798591	3.095314
treated crystals							**ug/ml**	
SW	59a	0.131	0.132	0.133	0.132	0.130	1.248799	
	59b	0.097	0.103	0.101	0.100	0.098	0.944605	
	59c	0.118	0.119	0.115	0.117	0.115	1.107909	1.100438
JQC	39a	0.046	0.047	0.049	0.047	0.045	0.435479	
	39b	0.018	0.019	0.020	0.019	0.017	0.163305	
	39c	0.030	0.030	0.031	0.030	0.028	0.272174	0.290319
ODY	40a	0.019	0.019	0.020	0.019	0.017	0.166507	
	40b	0.014	0.014	0.015	0.014	0.012	0.118476	
	40c	0.018	0.018	0.018	0.018	0.016	0.153698	0.146227
PN	41a	0.085	0.085	0.086	0.085	0.083	0.800512	
	41b	0.089	0.093	0.093	0.092	0.090	0.861351	
	41c	0.087	0.088	0.088	0.088	0.086	0.822927	0.828263
JT	42a	0.109	0.112	0.111	0.111	0.109	1.043868	
	42b	0.075	0.076	0.079	0.077	0.075	0.717259	
	42c	0.097	0.096	0.096	0.096	0.094	0.90618	0.889102
CYS	43a	0.079	0.080	0.080	0.080	0.078	0.746077	
	43b	0.079	0.081	0.083	0.081	0.079	0.758886	
	43c	0.079	0.080	0.080	0.080	0.078	0.746077	0.750347
treated cells								
SW	44a	0.020	0.021	0.021	0.021	0.019	0.179315	
	44b	0.021	0.021	0.021	0.021	0.019	0.182517	
	44c	0.021	0.021	0.021	0.021	0.019	0.182517	0.181449
JQC	45a	0.035	0.035	0.036	0.035	0.033	0.320205	
	45b	0.046	0.048	0.048	0.047	0.045	0.435479	
	45c	0.041	0.041	0.042	0.041	0.039	0.377842	0.377842
ODY	46a	0.049	0.049	0.049	0.049	0.047	0.451489	
	46b	0.074	0.073	0.074	0.074	0.072	0.688441	
	46c	0.062	0.063	0.063	0.063	0.061	0.582773	0.574234

PN	47a	0.047	0.047	0.046	0.047	0.045	0.429075	
	47b	0.047	0.046	0.047	0.047	0.045	0.429075	
	47c	0.047	0.047	0.046	0.047	0.045	0.429075	0.429075
JT	48a	0.095	0.096	0.096	0.096	0.094	0.899776	
	48b	0.068	0.071	0.072	0.070	0.068	0.65642	
	48c	0.083	0.083	0.081	0.082	0.080	0.771694	0.775963
CYS	49a	0.013	0.013	0.014	0.013	0.011	0.10887	
	49b	0.019	0.021	0.021	0.020	0.018	0.176113	
	49c	0.017	0.017	0.018	0.017	0.015	0.147294	0.144092
medium								
SW	50a	0.019	0.019	0.019	0.019	0.017	0.163305	
	50b	0.020	0.021	0.022	0.021	0.019	0.182517	
	50c	0.020	0.021	0.020	0.020	0.018	0.176113	0.173978
JQC	51a	0.007	0.007	0.007	0.007	0.005	0.048031	
	51b	0.038	0.039	0.037	0.038	0.036	0.345821	
	51c	0.025	0.025	0.024	0.025	0.023	0.217739	0.203864
ODY	52a	0.018	0.019	0.018	0.018	0.016	0.1569	
	52b	0.027	0.028	0.028	0.028	0.026	0.246558	
	52c	0.022	0.022	0.024	0.023	0.021	0.198527	0.200662
PN	53a	0.014	0.014	0.014	0.014	0.012	0.115274	
	53b	0.092	0.094	0.094	0.093	0.091	0.877362	
	53c	0.047	0.047	0.046	0.047	0.045	0.429075	0.473903
JT	54a	0.099	0.100	0.100	0.100	0.098	0.9382	
	54b	0.101	0.101	0.101	0.101	0.099	0.951009	
	54c	0.099	0.099	0.100	0.099	0.097	0.934998	0.941402
CYS	55a	0.127	0.128	0.129	0.128	0.126	1.210375	
	55b	0.031	0.031	0.031	0.031	0.029	0.278578	
	55c	0.080	0.079	0.081	0.080	0.078	0.74928	0.746077

ppm/ug

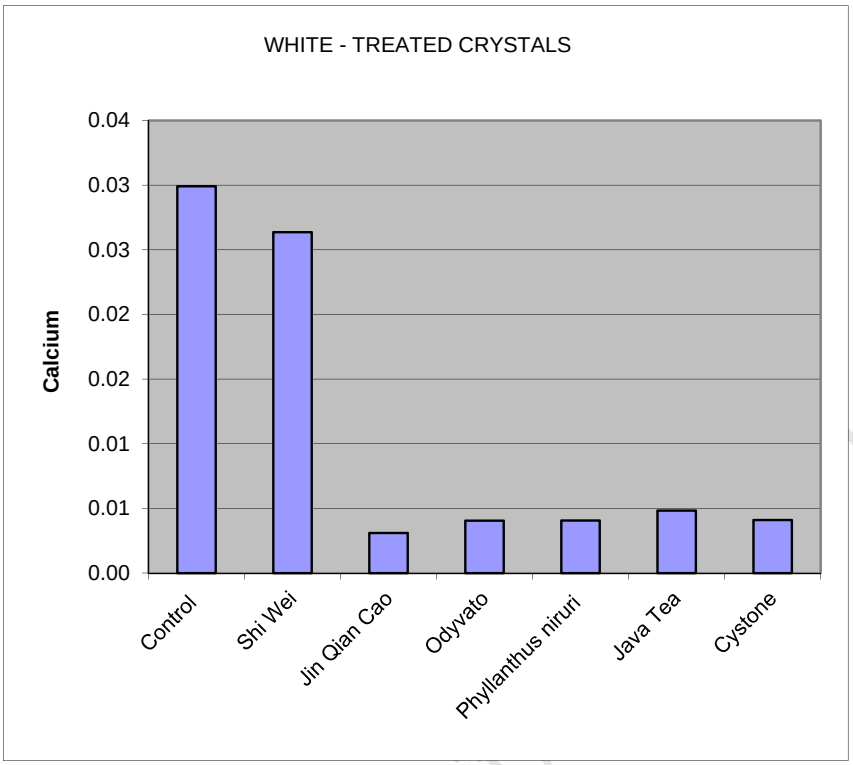
WHITE URINE

	crystals	cells	medium
Control	0.02991	0.02991	0.02991
Shi Wei	0.02636	0.00043	0.00164
Jin Qian Cao	0.00311	0.01007	0.00525
Odyvato	0.00405	0.00804	0.00233
Phyllanthus nir	0.00407	0.00179	0.00214
Java Tea	0.00484	0.01156	0.01149
Cystone	0.00411	0.00612	0.00461

ppm

WHITE URINE

	crystals	cells	medium
Control	3.0953	3.0953	3.0953
Shi Wei	1.1004	0.1814	0.1740
Jin Qian Cao	0.2903	0.3778	0.2039
Odyvato	0.1462	0.5742	0.2007
Phyllanthus r	0.8283	0.4291	0.4739
Java Tea	0.8891	0.7760	0.9414
Cystone	0.7503	0.1441	0.7461


WHITE - TREATED CRYSTALS
Calcium
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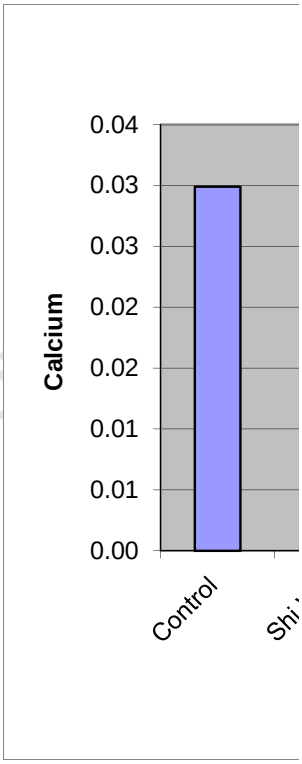
0.03
0.03
0.02
0.02
0.01
0.01
0.00
Control
Shi Wei
Jin Qian Cao
Odyvato
Phyllanthus niruri
Java Tea
Cystone


Calcium
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0.03
0.03
0.02
0.02
0.01
0.01
0.00
Control

mg/ml	per 2ml	%	average	protein	ppm/ug	
0.002715	0.0054307	0.27		171.821	0.0158	
0.003772	0.007544	0.38		71.592	0.0527	
0.002799	0.0055972	0.28	0.31	131.7297	0.0212	0.02991
mg/ml	**per 2ml**	**%**				
0.001249	0.0024976	0.12		46.535	0.0268	
0.000945	0.0018892	0.09		75.172	0.0126	
0.001108	0.0022158	0.11	0.11	27.921	0.0397	0.02636
0.000435	0.000871	0.04		318.5853	0.0014	
0.000163	0.0003266	0.02		57.98969	0.0028	
0.000272	0.0005443	0.03	0.03	52.97824	0.0051	0.00311
0.000167	0.000333	0.02		17.89805	0.0093	
0.000118	0.000237	0.01		205.470	0.0006	
0.000154	0.0003074	0.02	0.01	67.297	0.0023	0.00405
0.000801	0.001601	0.08		157.5029	0.0051	
0.000861	0.0017227	0.09		202.606	0.0043	
0.000823	0.0016459	0.08	0.08	285.653	0.0029	0.00407
0.001044	0.0020877	0.10		220.504	0.0047	
0.000717	0.0014345	0.07		169.674	0.0042	
0.000906	0.0018124	0.09	0.09	163.230	0.0056	0.00484
0.000746	0.0014922	0.07		131.0137	0.0057	
0.000759	0.0015178	0.08		255.584	0.0030	
0.000746	0.0014922	0.07	0.08	204.0378	0.0037	0.00411
0.000179	0.0003586	0.02		539.805	0.0003	
0.000183	0.000365	0.02		365.836	0.0005	
0.000183	0.000365	0.02	0.02	386.598	0.0005	0.00043
0.00032	0.0006404	0.03		40.80756	0.0078	
0.000435	0.000871	0.04		81.615	0.0053	
0.000378	0.0007557	0.04	0.04	22.194	0.0170	0.01007
0.000451	0.000903	0.05		32.93242	0.0137	
0.000688	0.0013769	0.07		110.252	0.0062	
0.000583	0.0011655	0.06	0.06	139.605	0.0042	0.00804

0.000429	0.0008581	0.04		718.0699	0.0006	
0.000429	0.0008581	0.04		137.457	0.0031	
0.000429	0.0008581	0.04	0.04	259.880	0.0017	0.00179
0.0009	0.0017996	0.09		74.4559	0.0121	
0.000656	0.0013128	0.07		65.149	0.0101	
0.000772	0.0015434	0.08	0.08	61.569	0.0125	0.01156
0.000109	0.0002177	0.01		12.17068	0.0089	
0.000176	0.0003522	0.02		37.944	0.0046	
0.000147	0.0002946	0.01	0.01	30.78465	0.0048	0.00612
0.000163	0.0003266	0.02		47.967	0.0034	
0.000183	0.000365	0.02		280.641	0.0007	
0.000176	0.0003522	0.02	0.02	204.038	0.0009	0.00164
4.8E-05	9.606E-05	0.005		291.3803	0.0002	
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0.000877	0.0017547	0.09		160.367	0.0055	
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0.000938	0.0018764	0.09		37.22795	0.0252	
0.000951	0.001902	0.10		231.959	0.0041	
0.000935	0.00187	0.09	0.09	180.412	0.0052	0.01149
0.00121	0.0024207	0.12		133.1615	0.0091	
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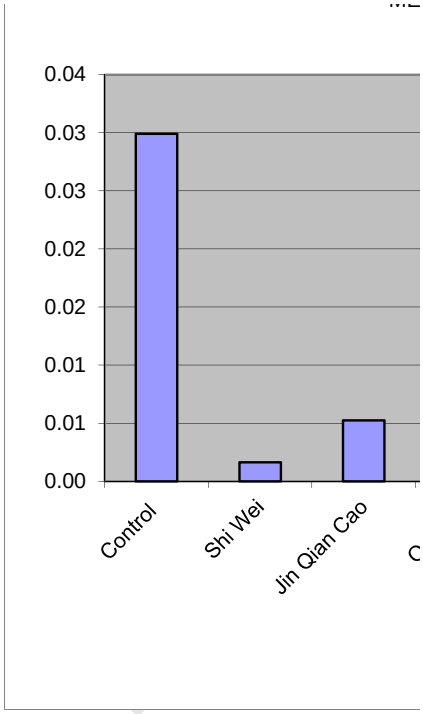

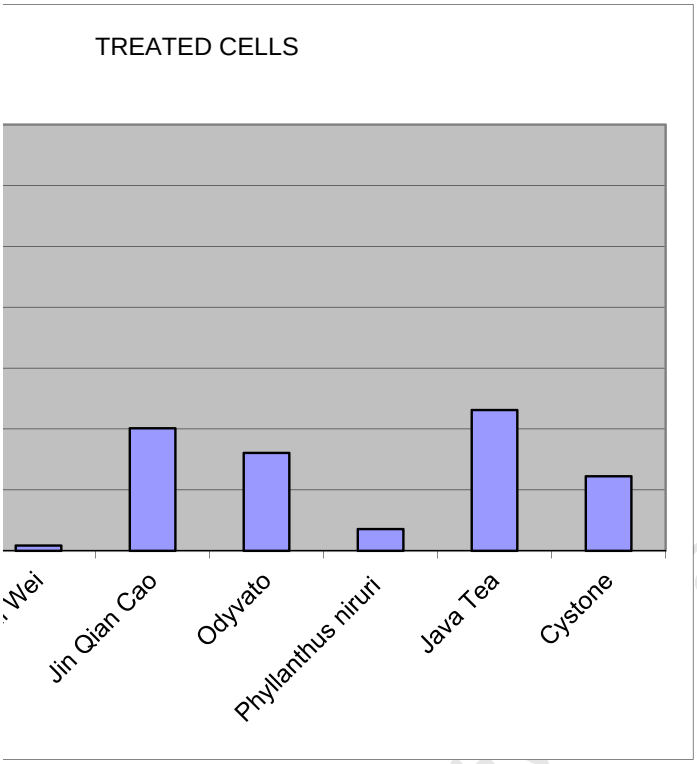

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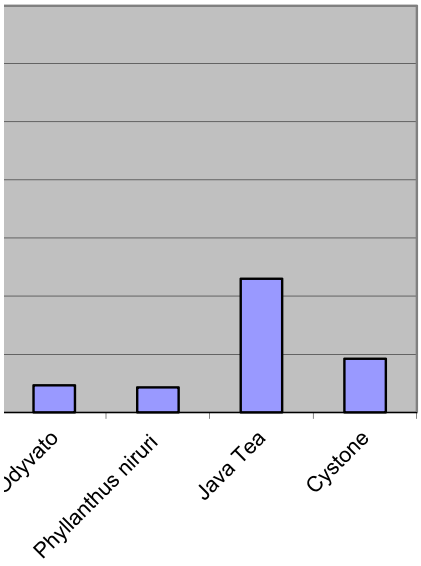

Calcium

University of Cape Town

Department of Chemistry

Information Sheet

Project Title: Investigation of the effects of traditional medicines on kidney stone risk factors

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Urinary stones are a frequent problem affecting many individuals. Prophylaxis and shock wave therapy are some common methods of treatment, but there is a high rate of recurrence due to no effective cure being known. Chinese herbal preparations have been used to treat renal stones for many years.

Researchers at the University of Cape Town Kidney Stone Research Laboratory are interested in studying the inhibitory effects of traditional Chinese herbs on the formation of calcium oxalate stones *in vivo*. You are invited to participate in a study conducted by Ms Ronica Ramsout, PhD student in the Department of Chemistry under the supervision of Professor AL Rodgers, Head of the Department of Chemistry and Director of the Kidney Stone Research Laboratory.

You will be required to ingest 3 capsules containing a Chinese traditional herb *Shi Wei*, after breakfast everyday for 7 consecutive days. On these days, you need to avoid calcium and oxalate rich foods, and you will have to provide a 24 hour urine sample for analysis. We shall closely monitor your health for side effects. The possible adverse effects of these compounds are diarrhoea, abdominal pain, vomiting and nephritis **but none have been reported in previous studies**. Baseline creatinine and liver function tests (monitoring of transaminases) will be performed on all subjects. Urinary creatinine levels will be monitored daily as a safety marker i.e. creatinine levels must not become elevated. Transaminase levels will be monitored on days 0, 3 and 7. Other liver function tests will be performed based on results of transaminase levels and urinary creatinine. Blood will be drawn by trained phlebotomists from the Pathcare Laboratory and analysis will be carried out within 24 hours.

Dr Dick Barnes, a medically trained professional, is affiliated to this study and any symptoms can be reported to him. Dr Barnes's contact details are: Dick.Barnes@uct.ac.za, 021 404 6105, Department of Urology, Ward E26, Groote Schuur Hospital. This study will be covered by the UCT no-fault liability insurance cover and participants will be compensated in the event of any injuries being done to them.

Prior to analysis you are required to provide a 24 hour baseline urine sample for analysis. You will also need to fill out a health questionnaire and answer some general questions with respect to your medical history.

There is no payment or reward for participating in this study, but you will be reimbursed for petrol costs. Urine results can be made available to you should you be interested to know them. You may withdraw from this study at anytime without any penalty or having to give a reason. We can also exclude you from this investigation if you do not comply with the instructions requested of you.

All information related to you will remain confidential and will be identified by codes known only to the investigator. The results will be made available to the scientific community but no names will be linked to it.

If you interested in being a participant in this study or would like any information, please contact Ms Ronica Ramsout on 021 650 2534 or 084 472 9223.

If you have any queries about your rights or welfare as a research participant, please contact Professor Marc Blockman, Chairperson of the Research Ethics Committee in the Faculty of Health Sciences (Tel No.: 021 406 6889).

Patient Consent Sheet

I _____ (*Name and Surname*), have carefully read and understood the Patient Information Sheet and Informed Consent. All my questions about the study were answered to my complete satisfaction and I can keep copies of the written Patient Information Sheet and Informed Consent. I was given enough time to decide about taking part in this study.

I have been informed of the risks and benefits of this investigation. I understand that the information obtained from this research may be used in future research and be published; however my right to privacy will be maintained.

By signing this Informed Consent Sheet (*to be signed by parents if participant is under 21 years of age*), I declare that I take part in this study of my own free will and without being persuaded or pressured by any other person. I am aware that I am liberty to withdraw from this study at any time, without giving a reason, but I will not be reimbursed for travel expenses or inconveniences.

I document my informed consent by signing my name below:

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Participant/Parent Signature Date

I _____ (Name of Researcher) have fully informed the above person of the procedures, aims and risks of the study and answered questions truthfully.

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Researcher Signature Date

Health Questionnaire

Identification Code _____

General Info

Date _____

Full Name _____

Address _____

Email _____

Date of Birth _____

Telephone No. _____

Nutritional/Exercise Info

Weight _____kg__

Height _____m___

Do you follow any special diet (e.g. vegetarian)? _____

If yes, please specify _____

Are you taking any vitamin/mineral supplements? _____

If yes, please give details (name, dose, frequency) _____

What kind of exercise do you do? _____

Hours per week _____

Exposure to toxic substances

Do you smoke? _____

If yes, how many a day? _____

Do you consume alcohol? _____

If yes, how many a week? _____

(1 drink = 1 can of beer or 1 glass of wine)

Medical history

Have you had any illnesses lately? _____

If yes, please give details

_____

Have you undergone any surgery in the last year? _____

If yes, please give details

_____

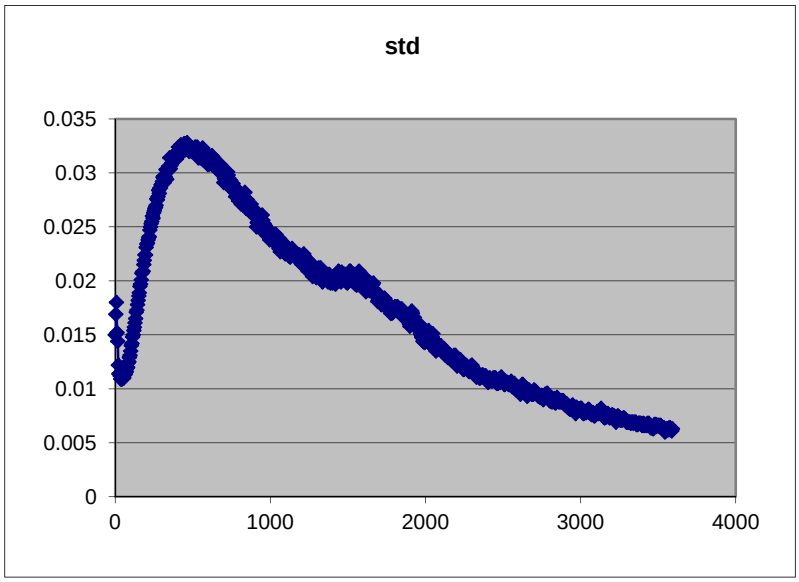
Do you take any medication? _____

If yes, please give details (prescription or over-the-counter drug, frequency, dose)

_____

____

Have you taken any antibiotics in the last year? __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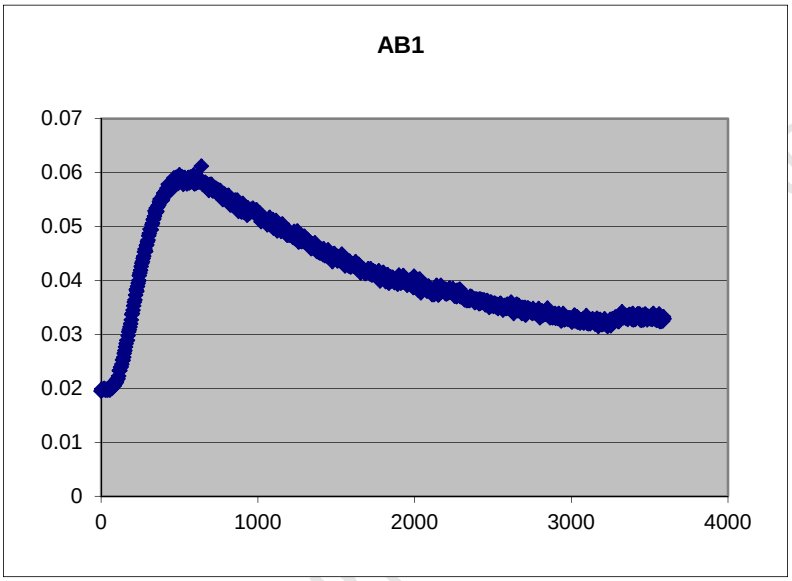
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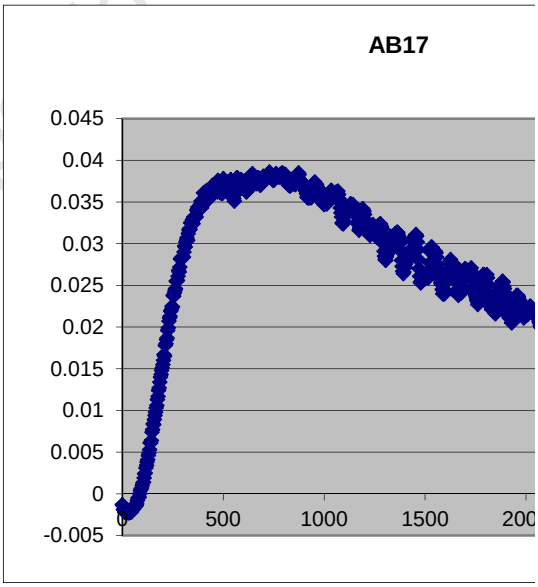
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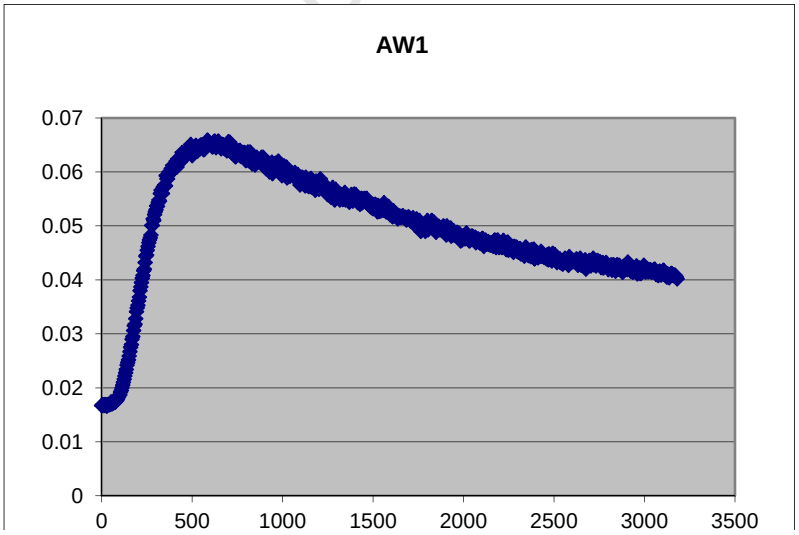
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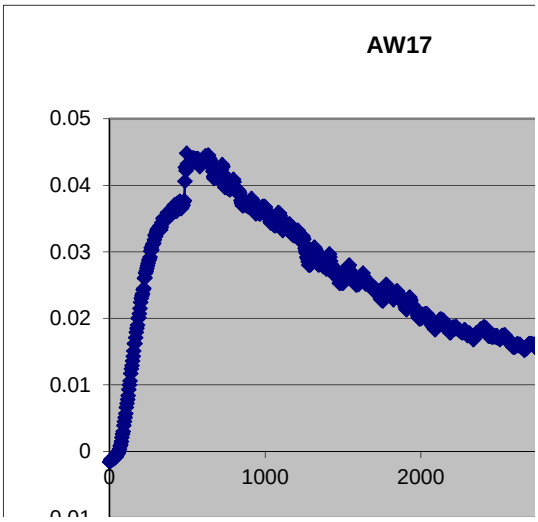
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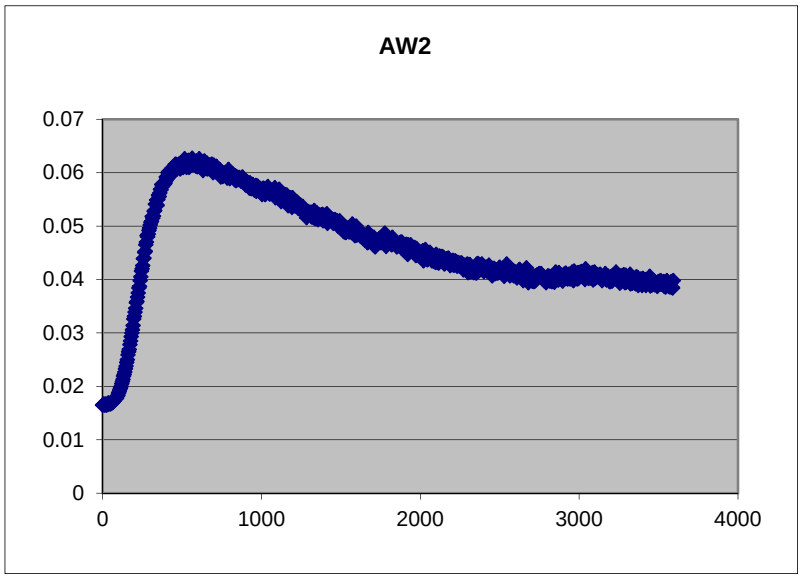

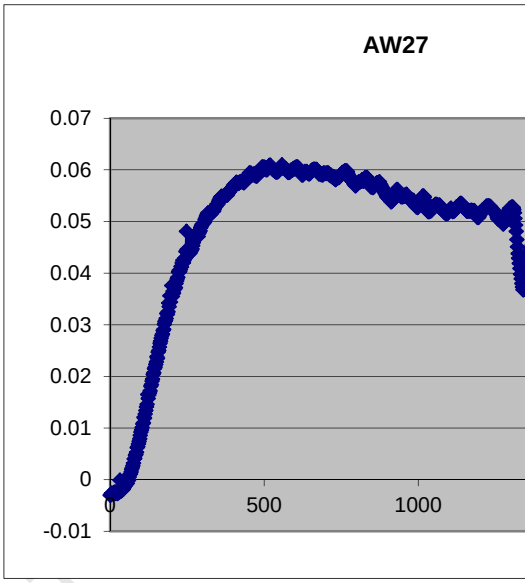

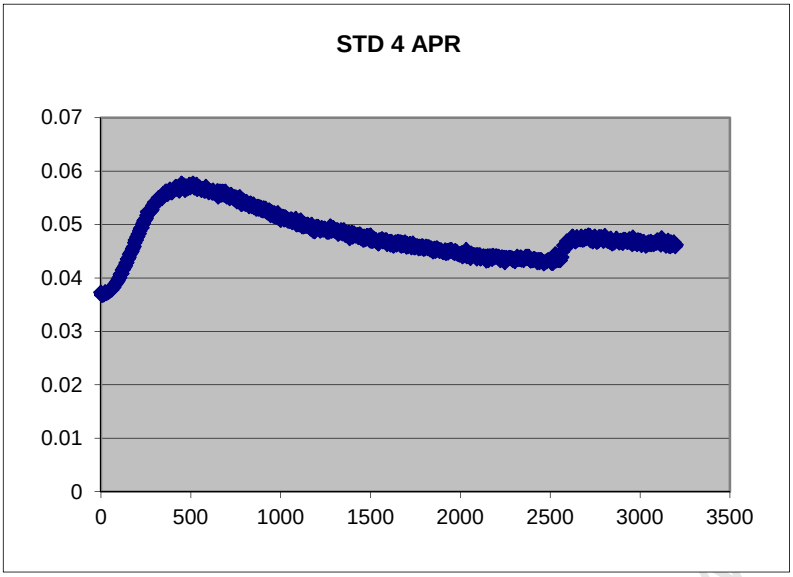
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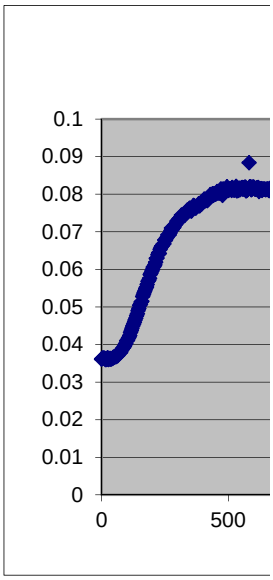
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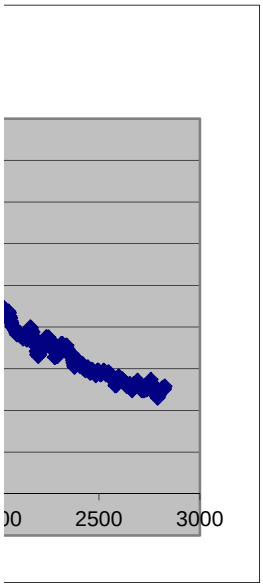
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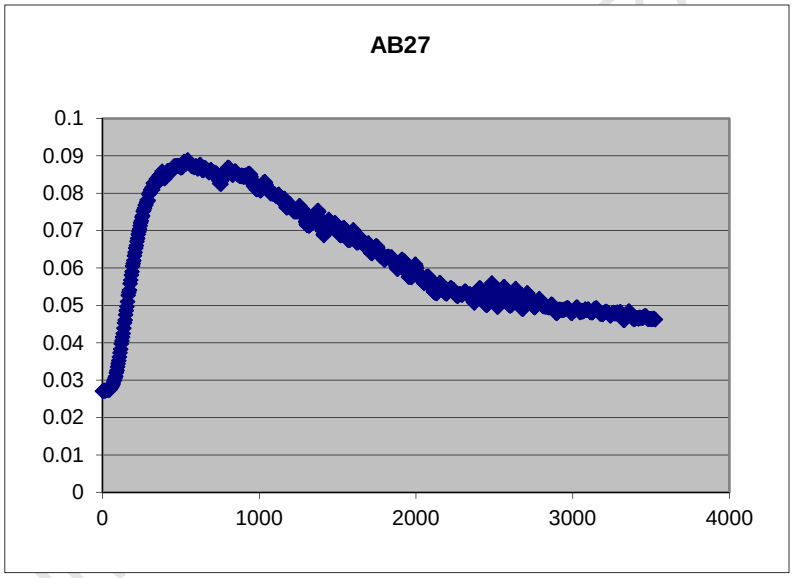
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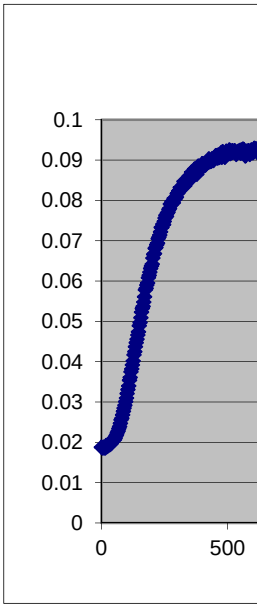
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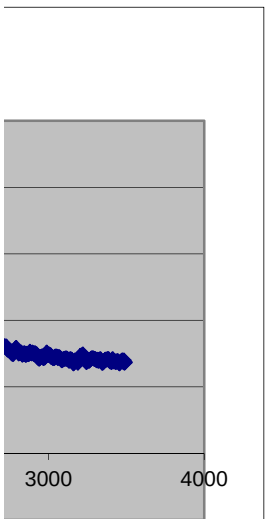
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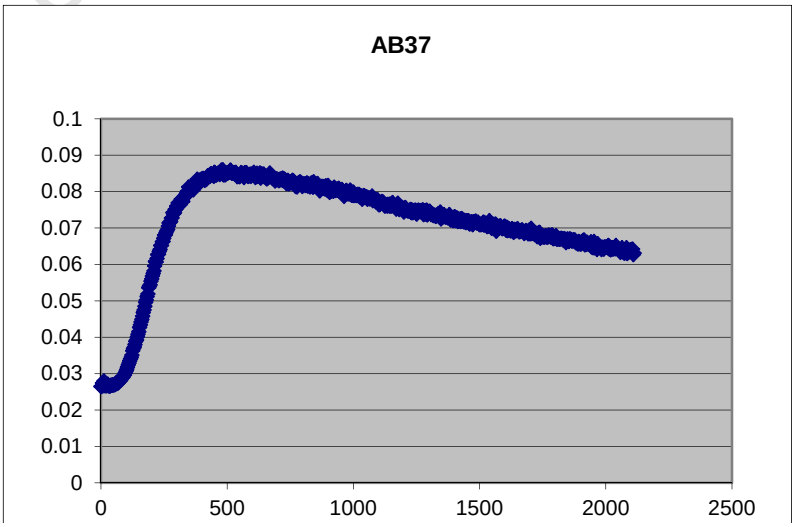
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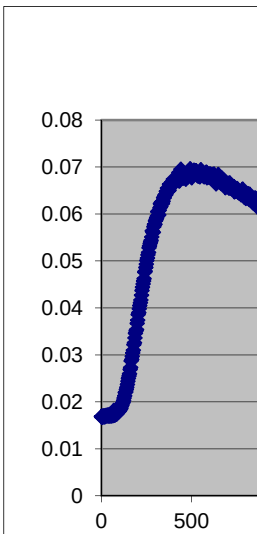
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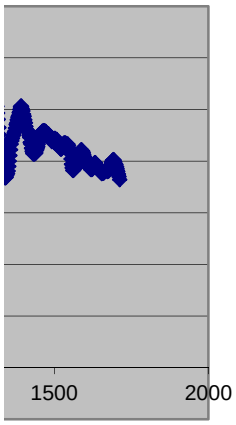

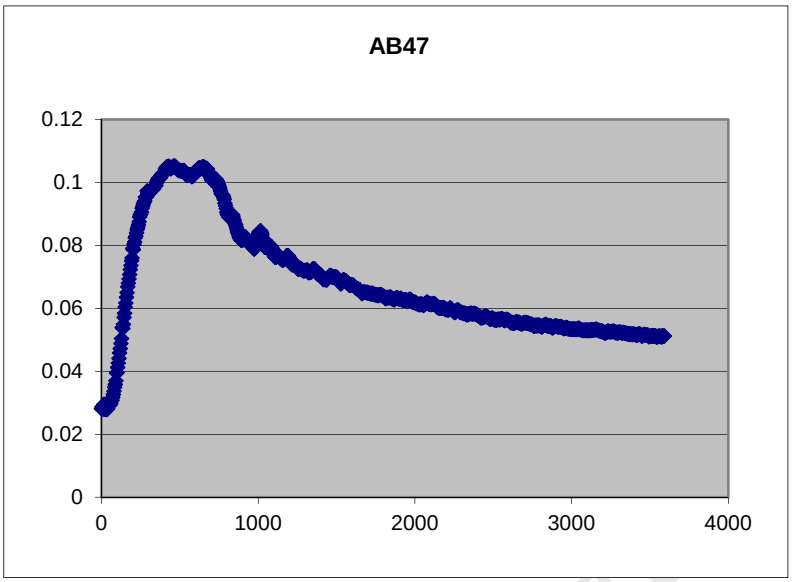

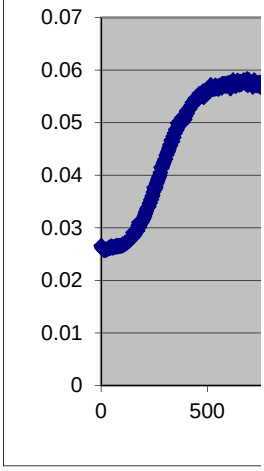

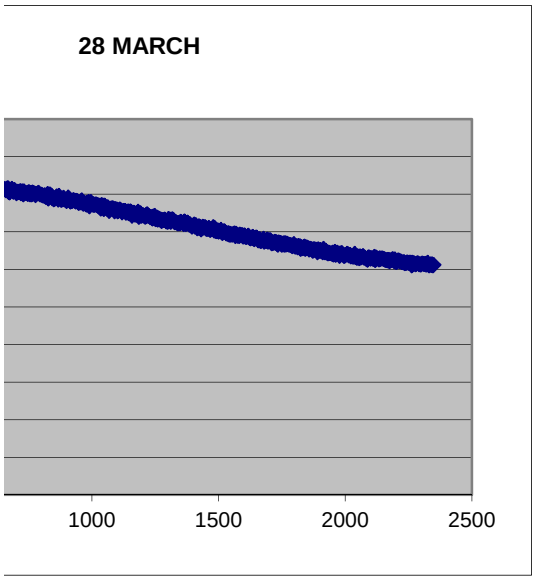
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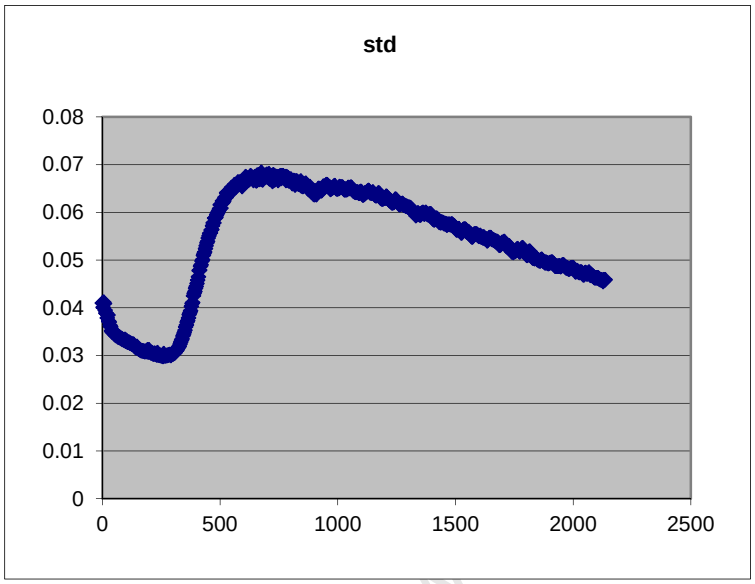
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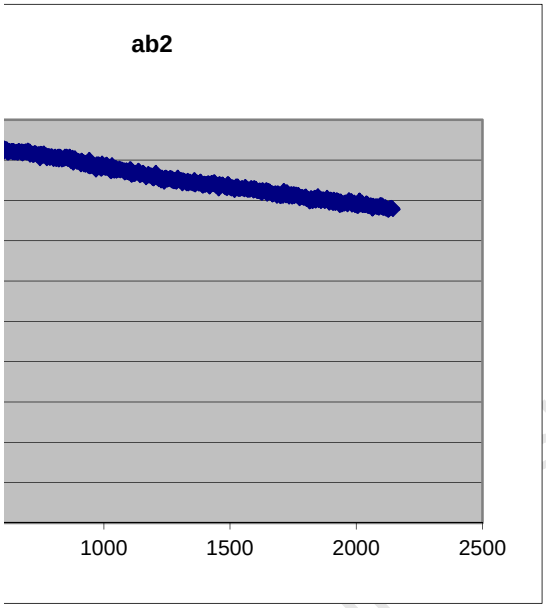
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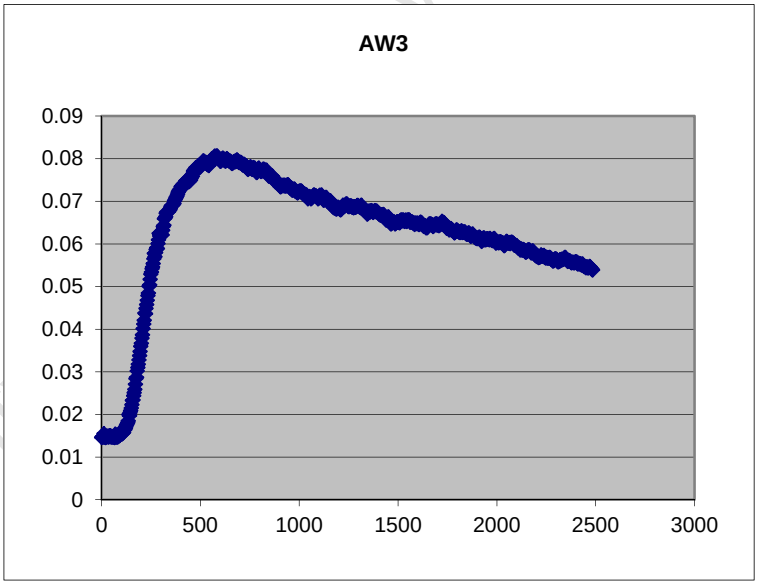
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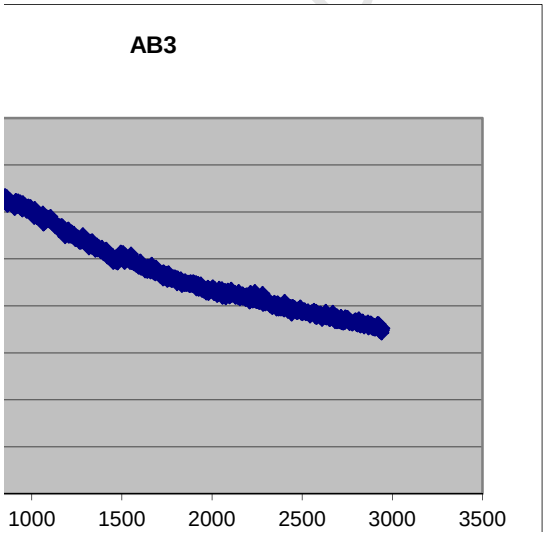
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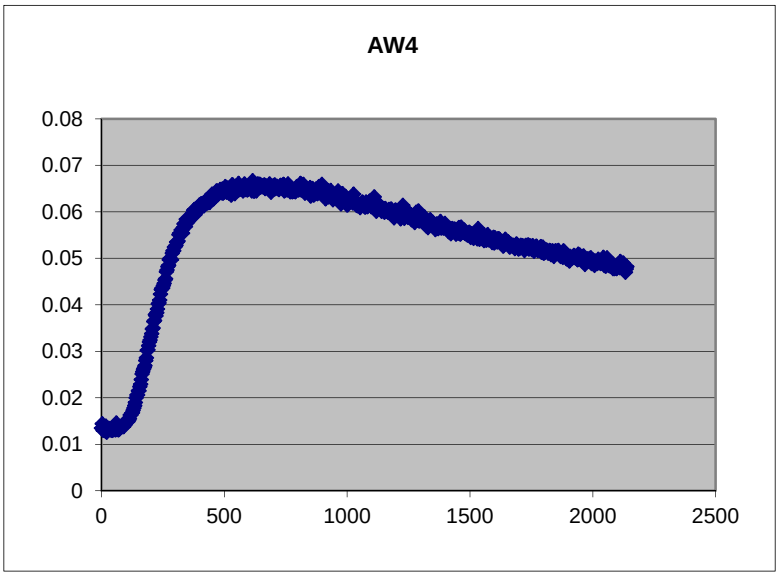
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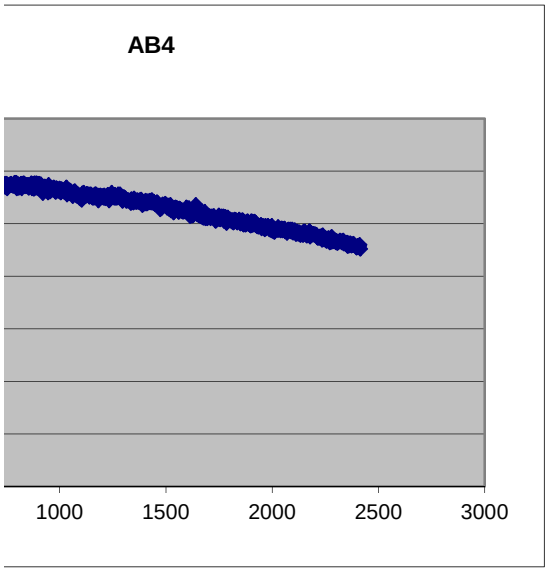
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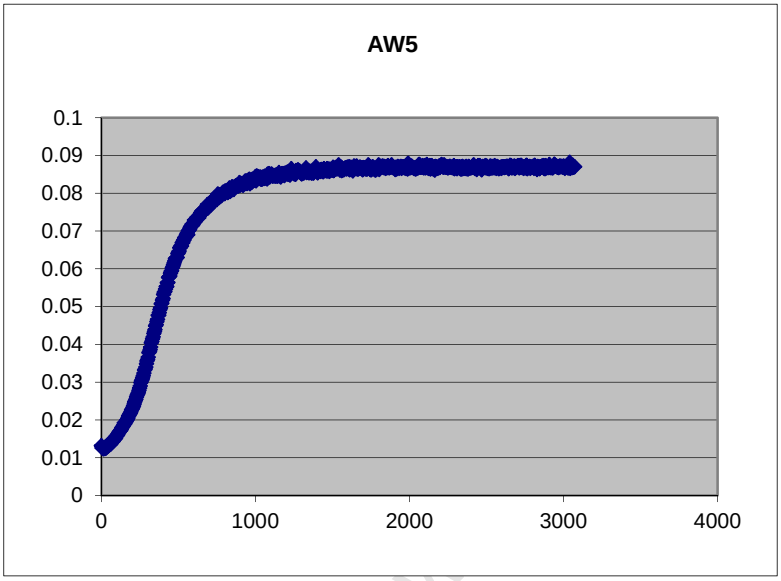
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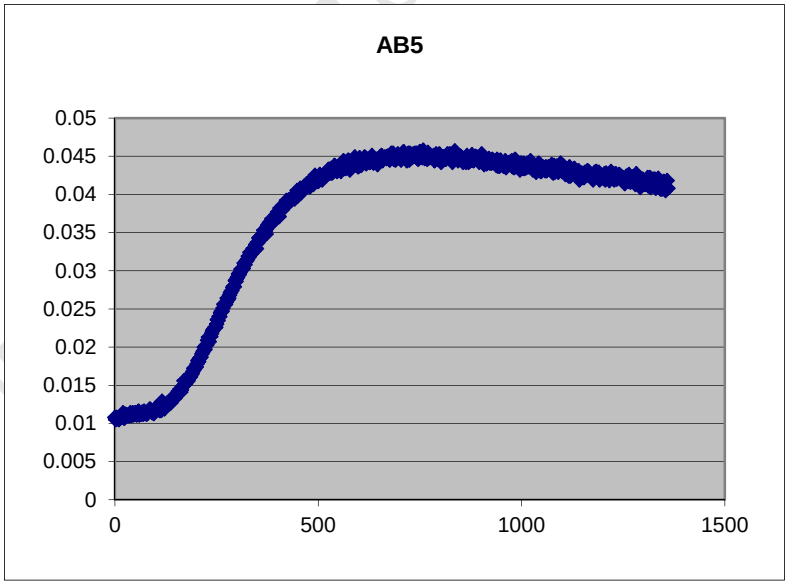
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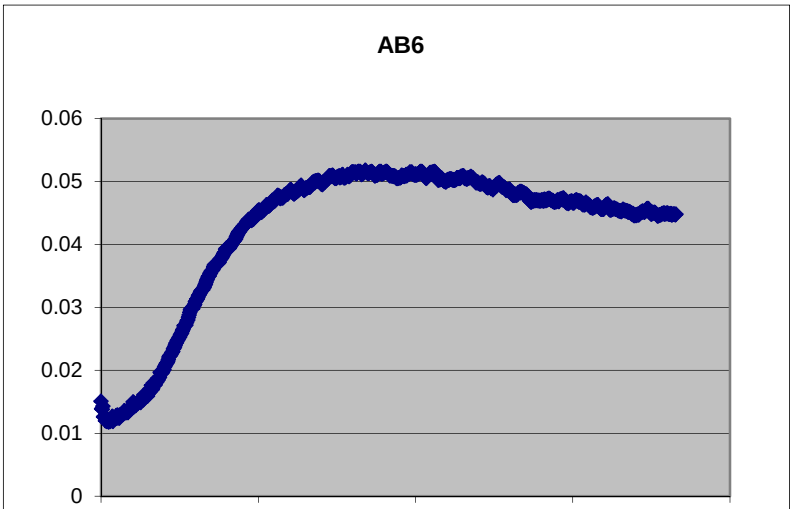
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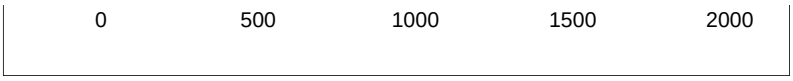

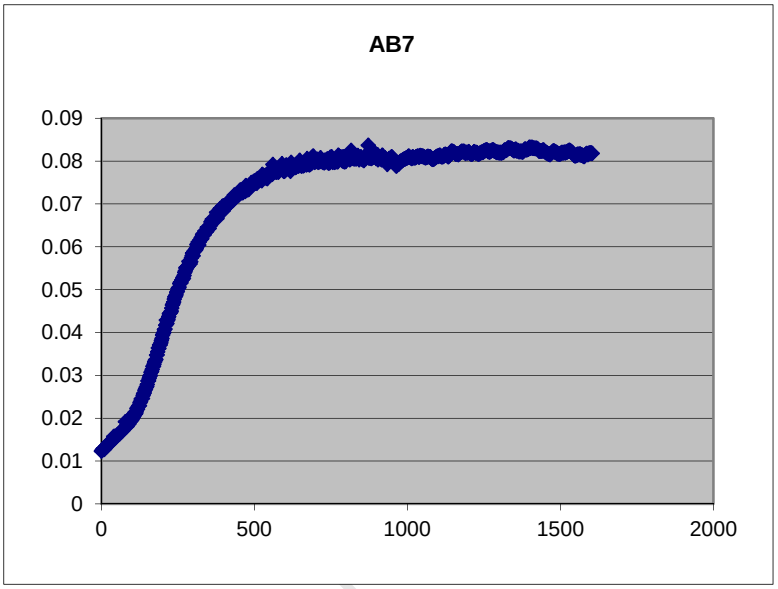

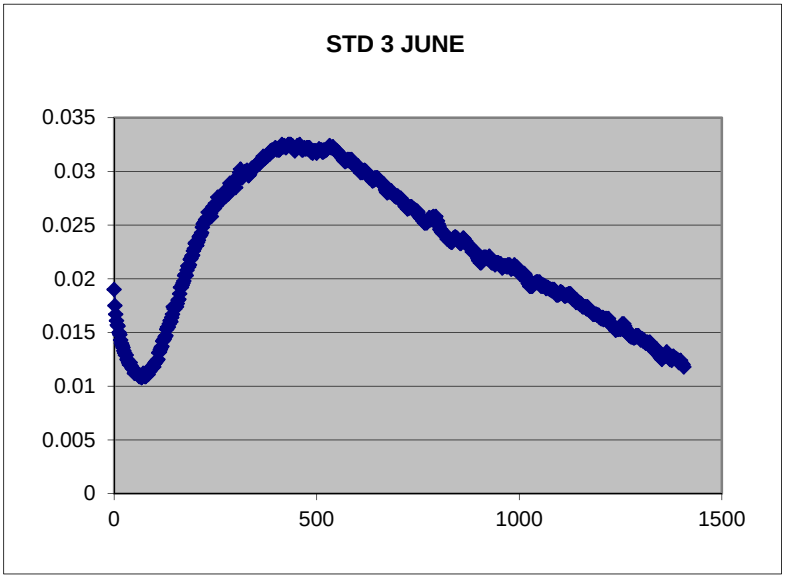
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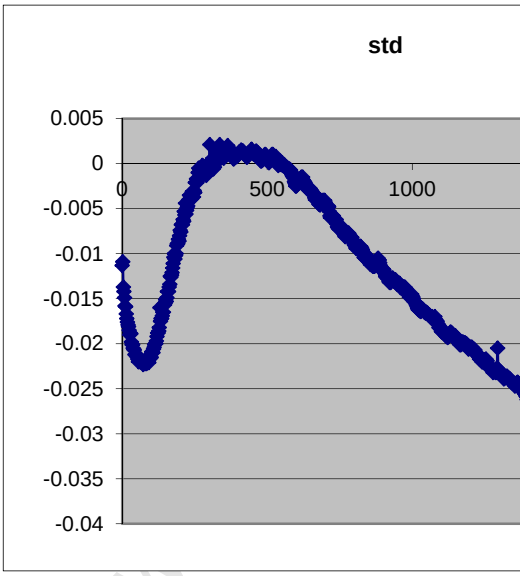
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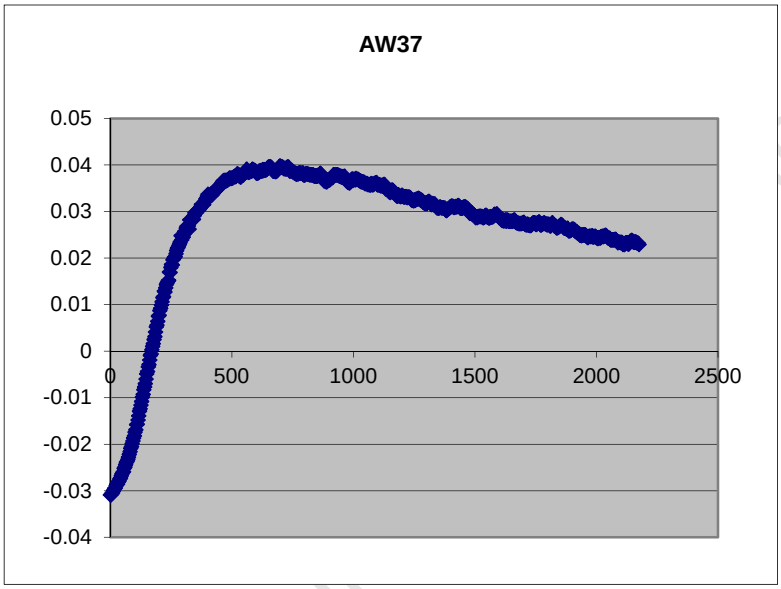
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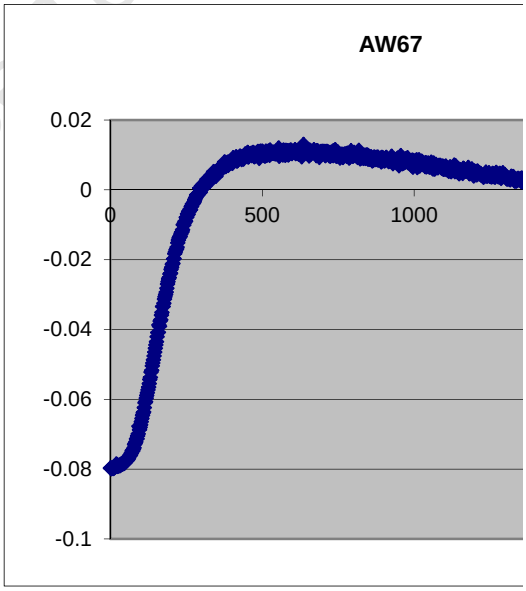
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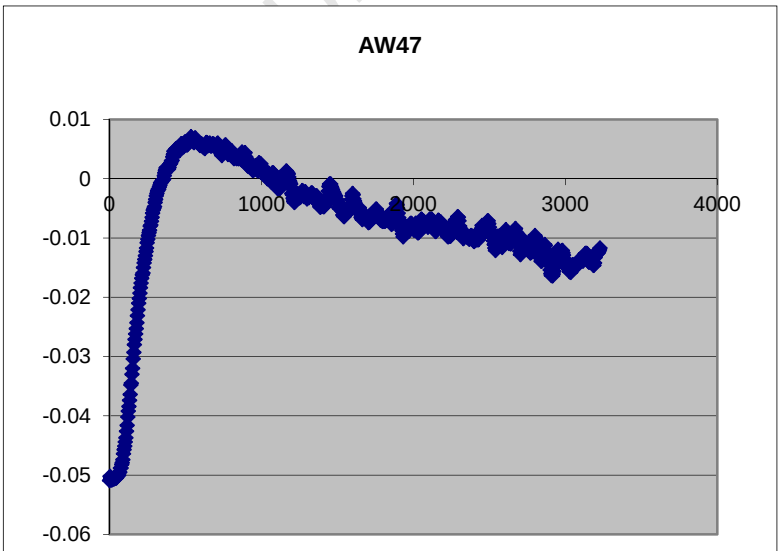
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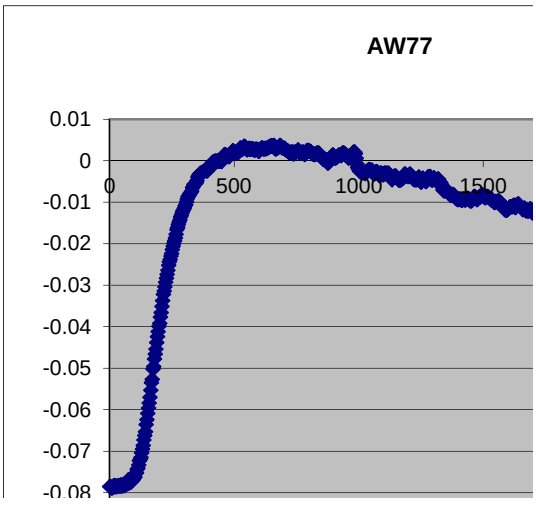
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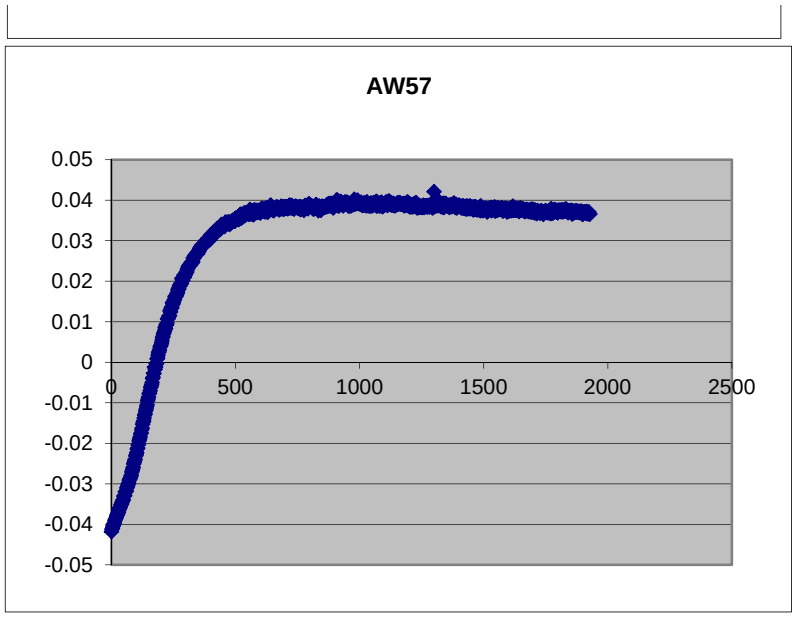
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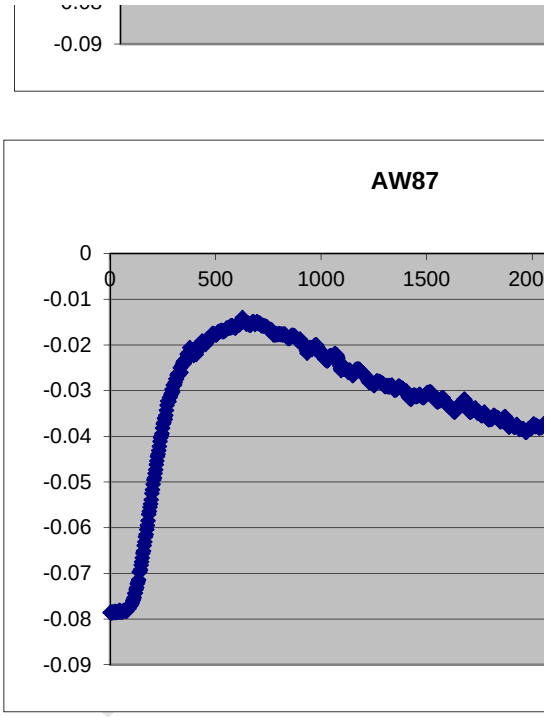
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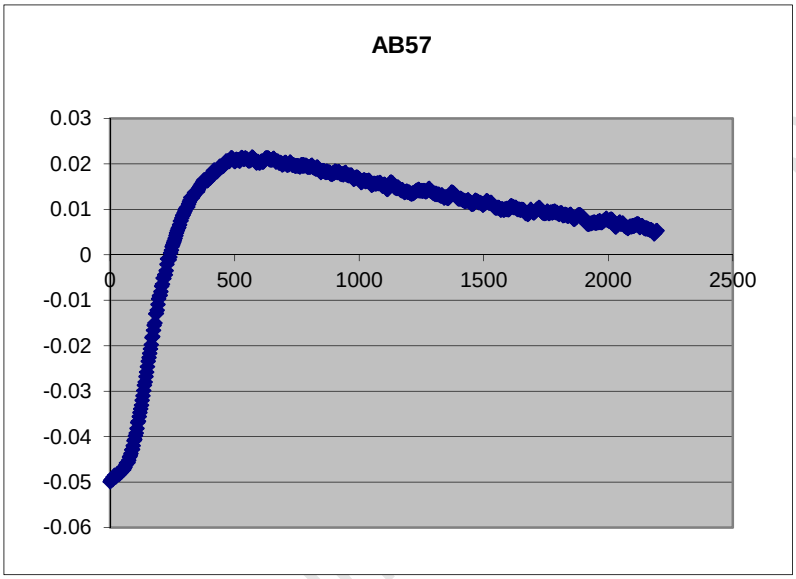
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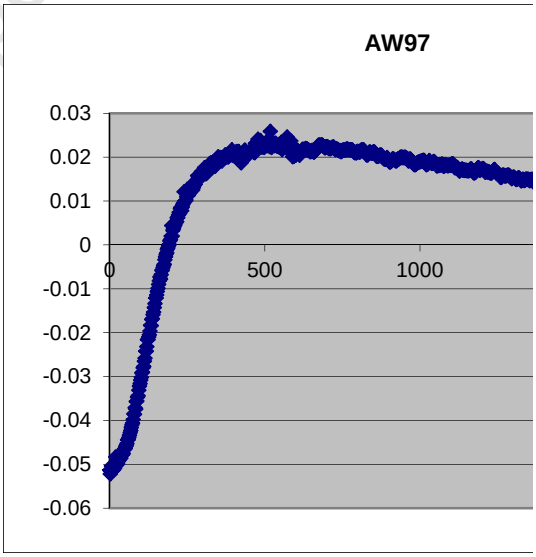
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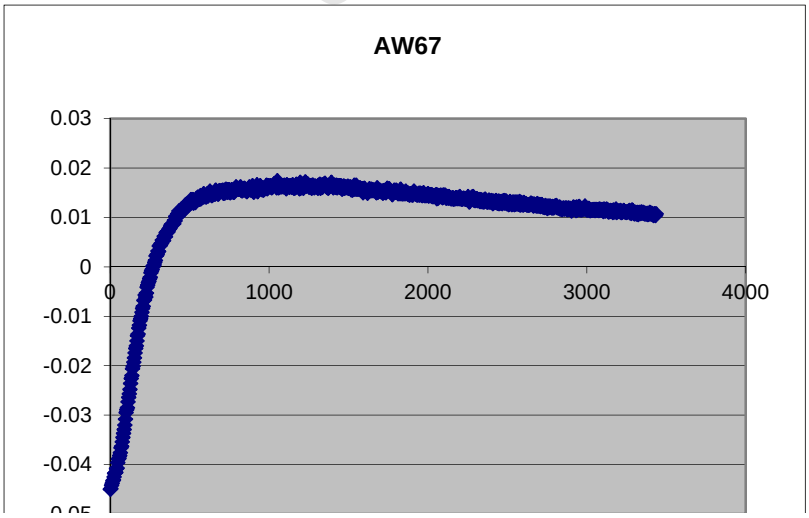
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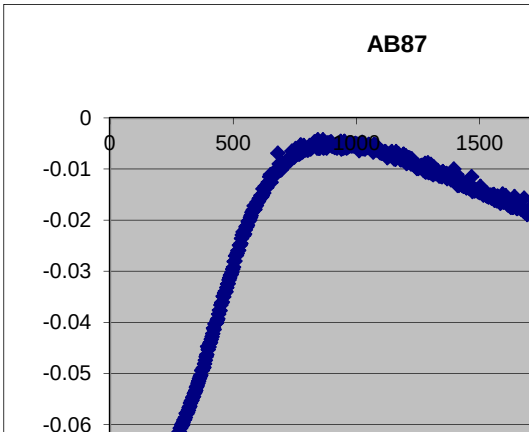
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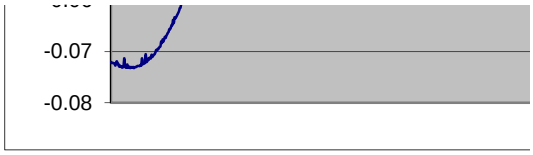

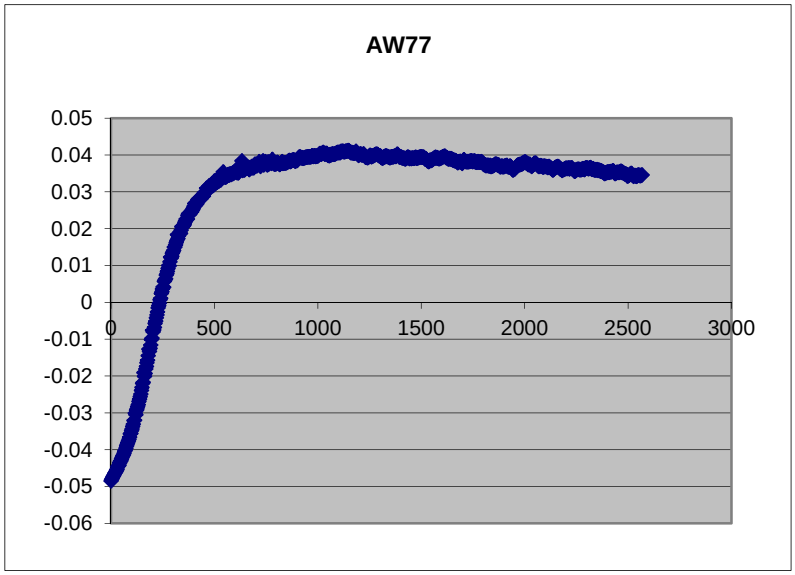

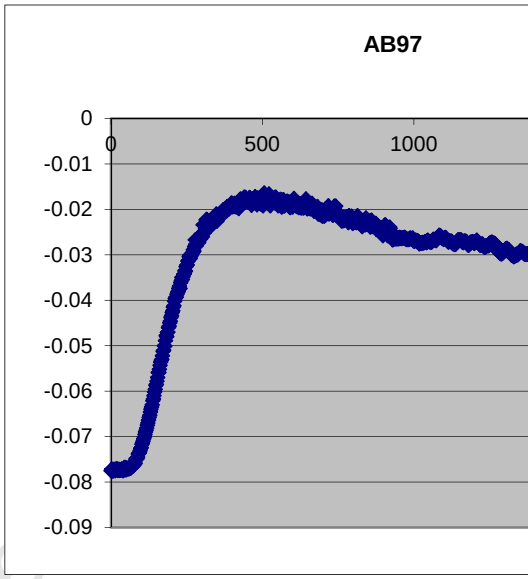

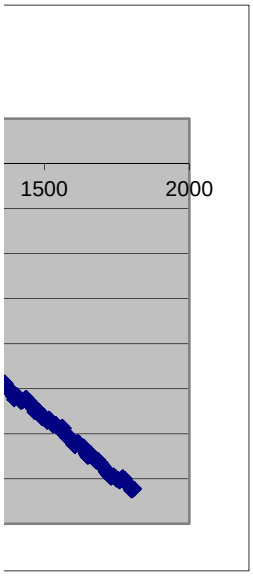
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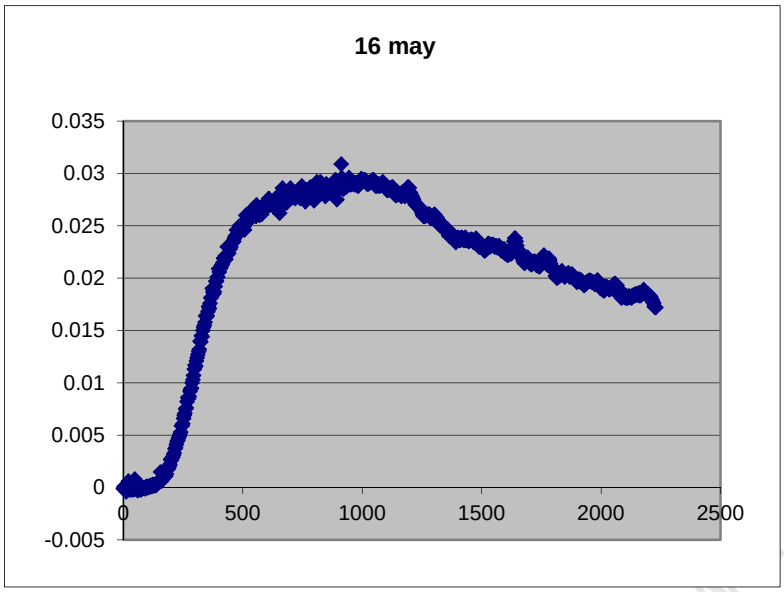
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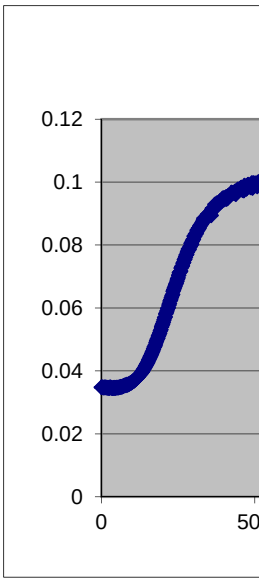
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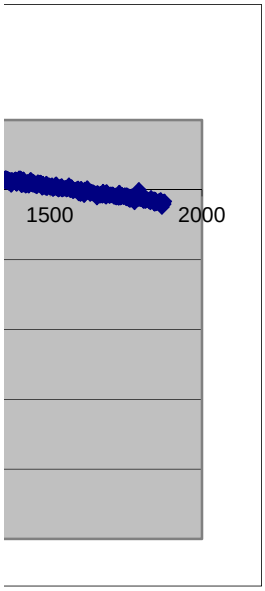
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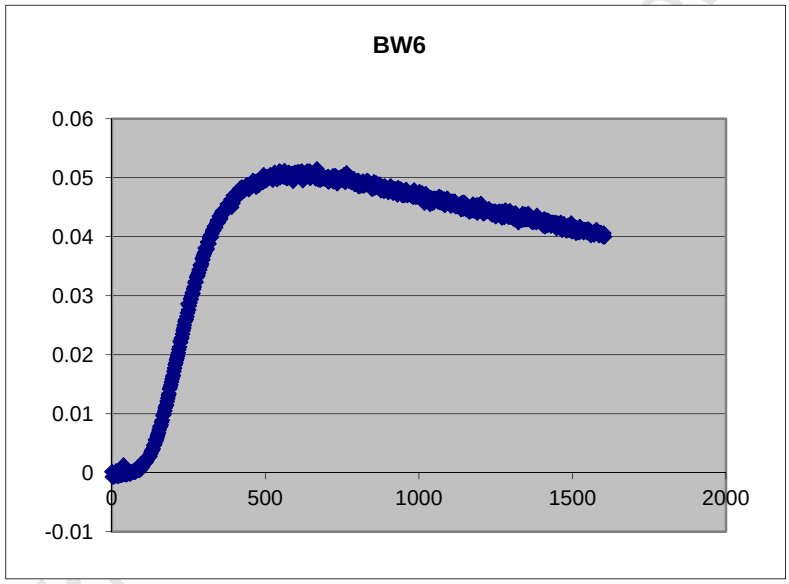
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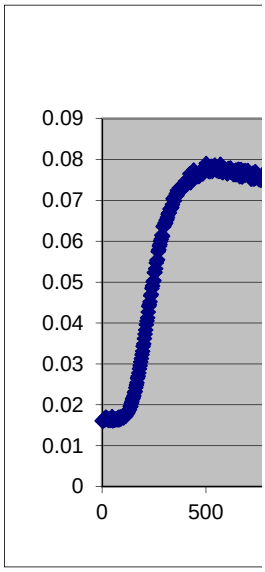
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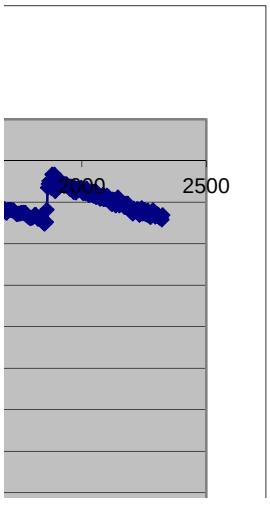
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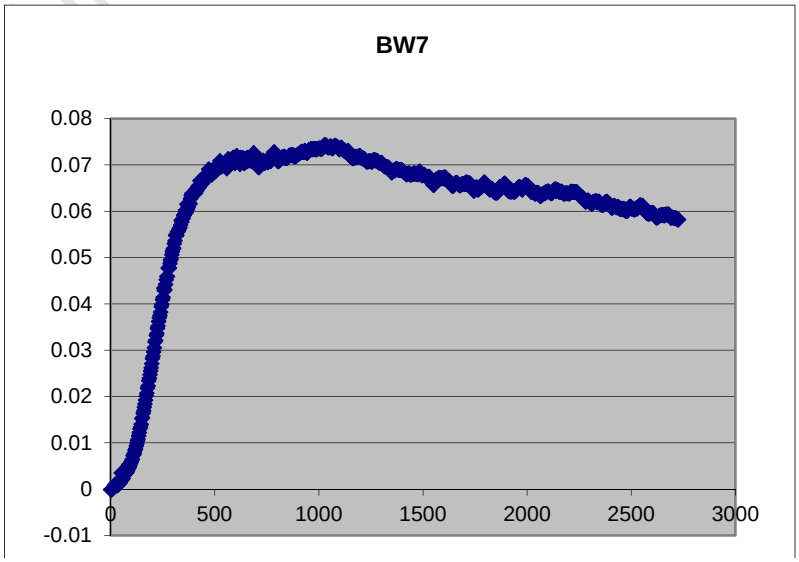
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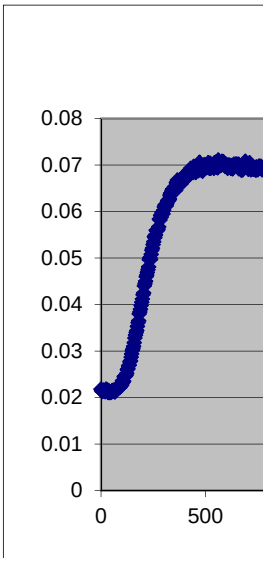
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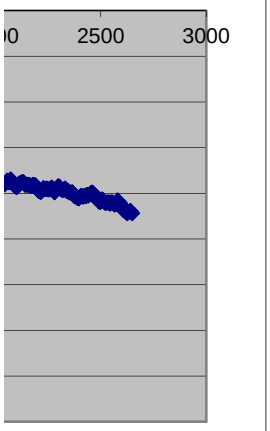
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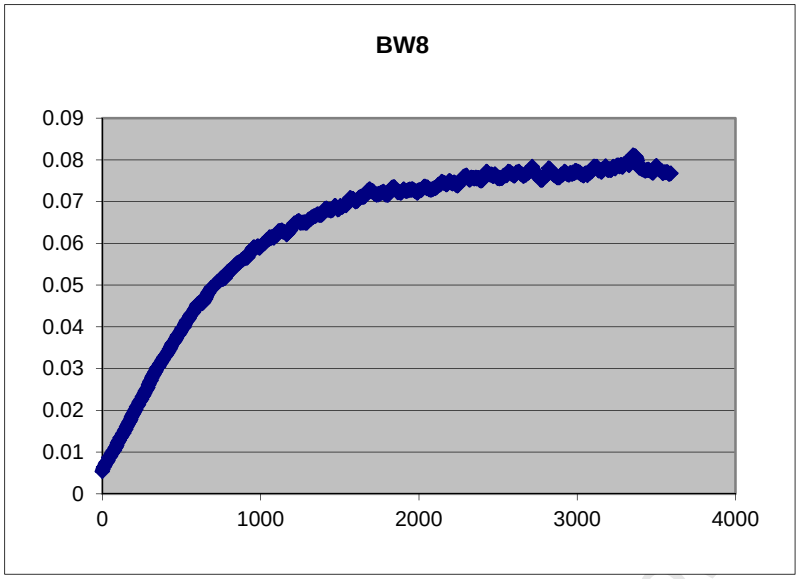
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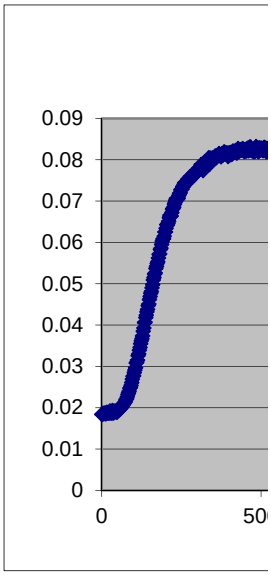
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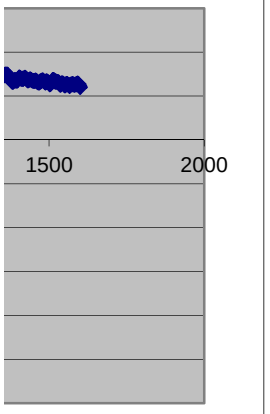
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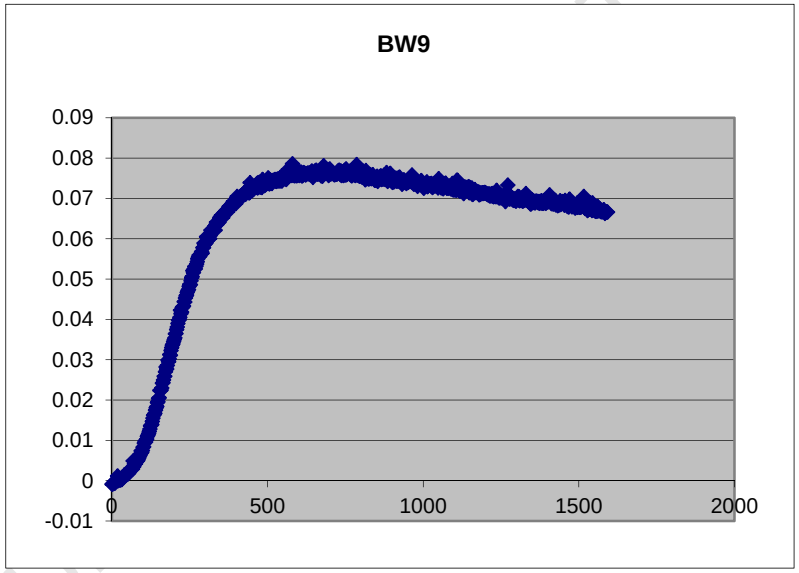
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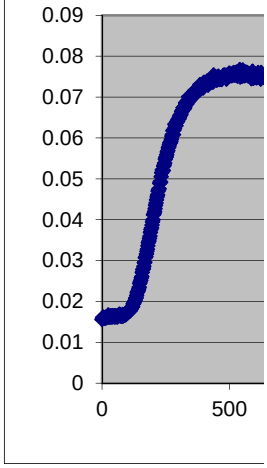
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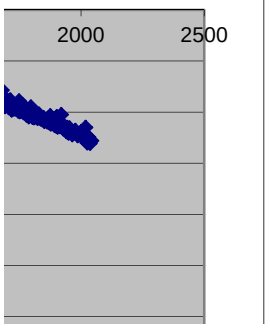
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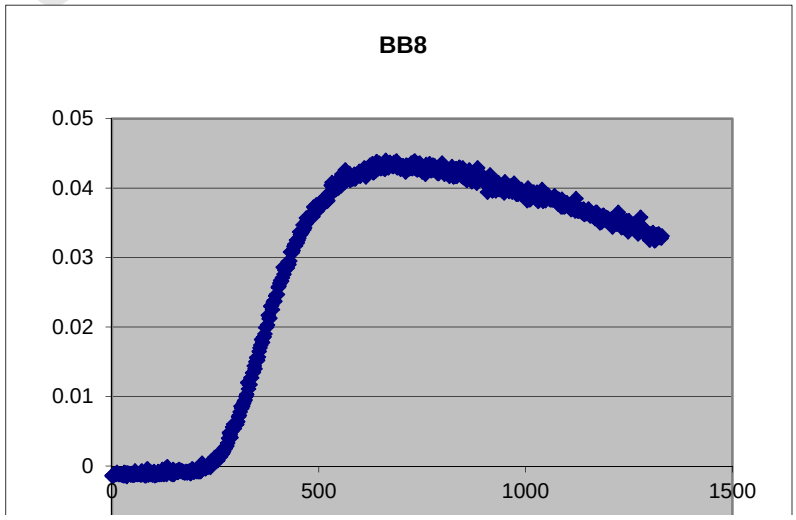
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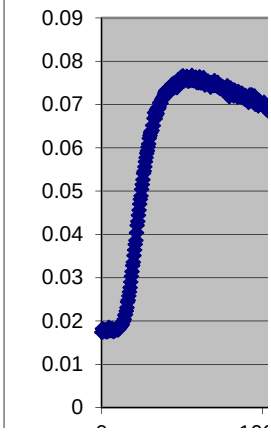
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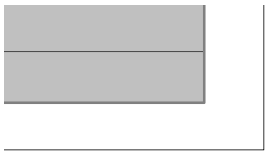

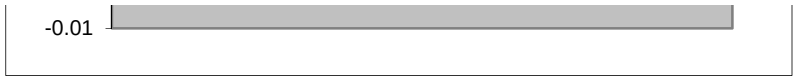

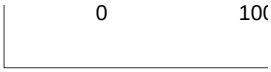

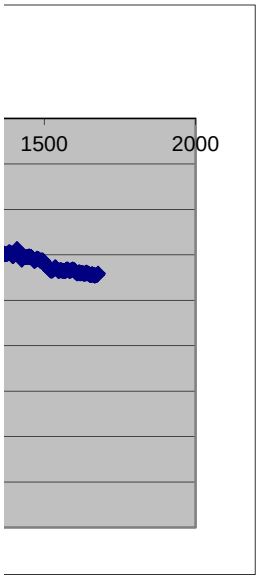

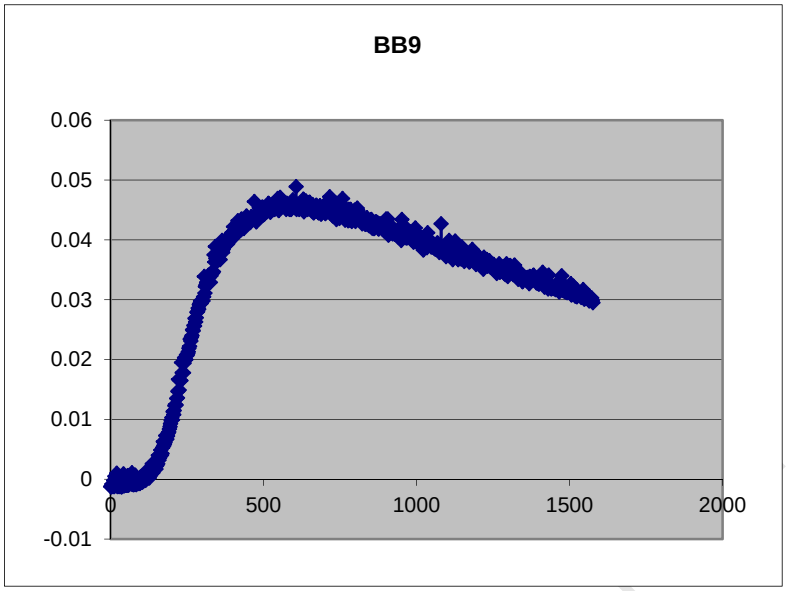

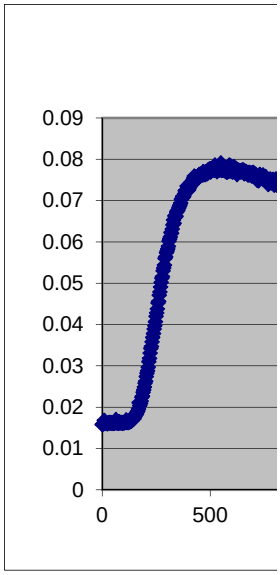

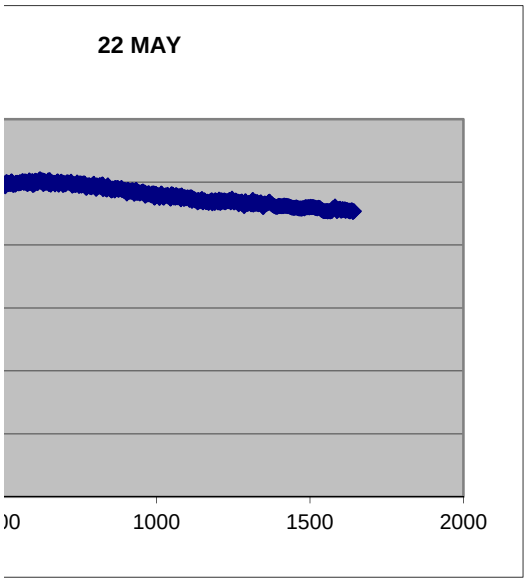
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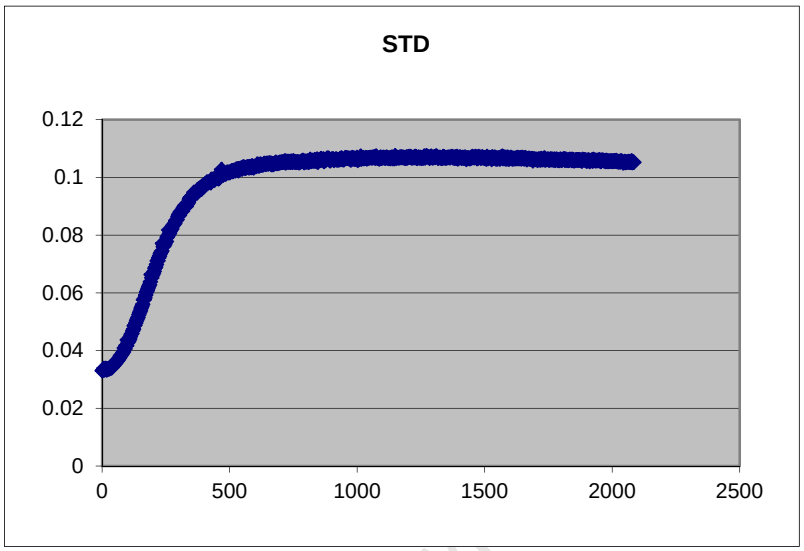
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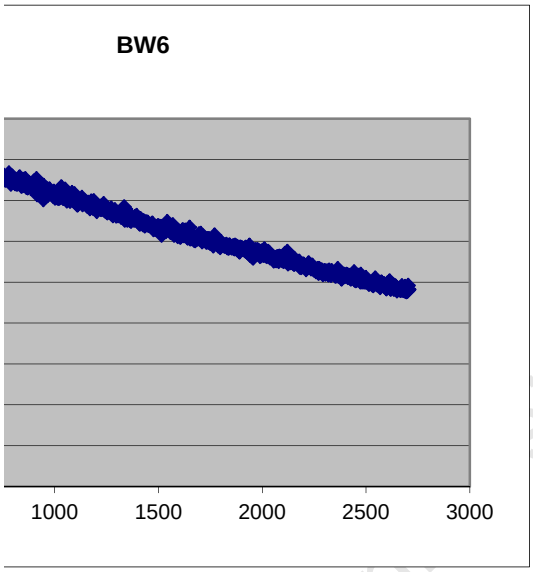
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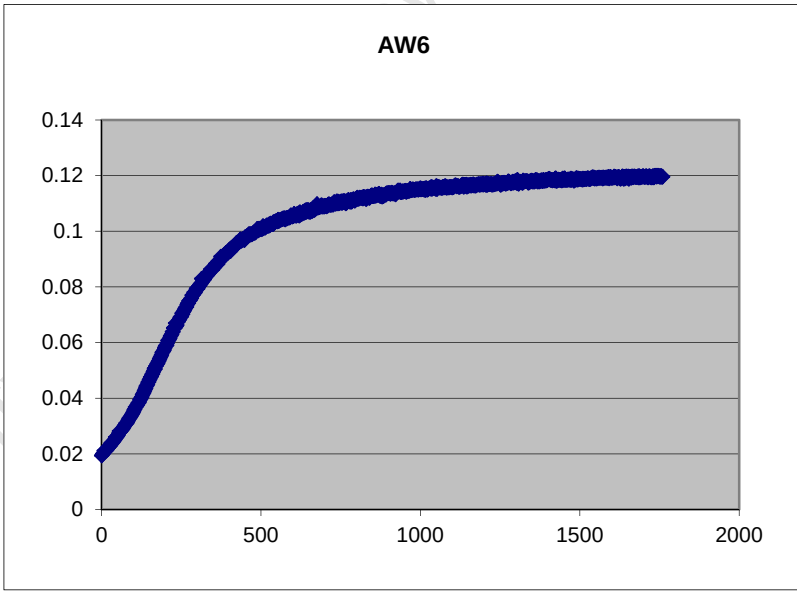
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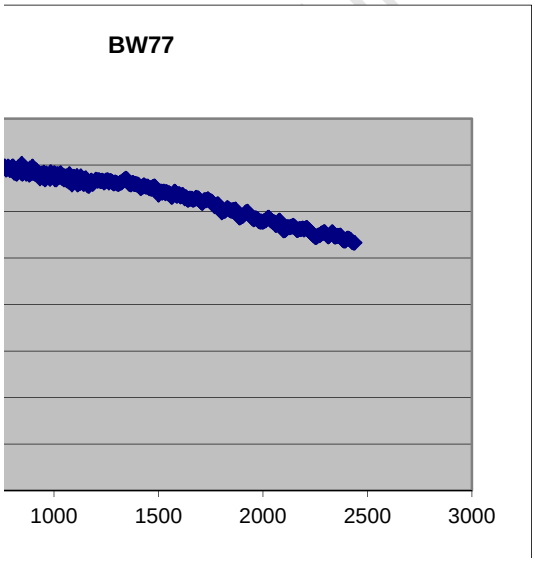
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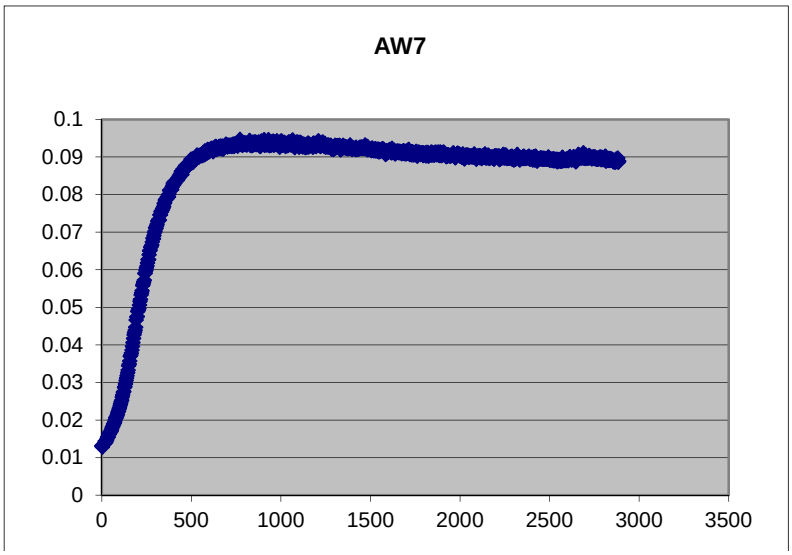
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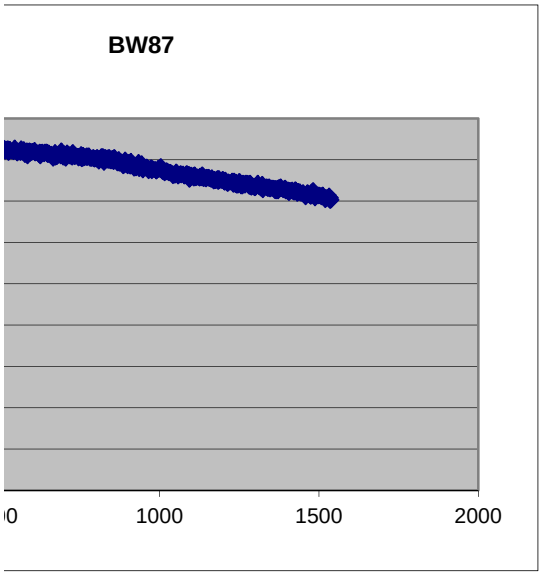
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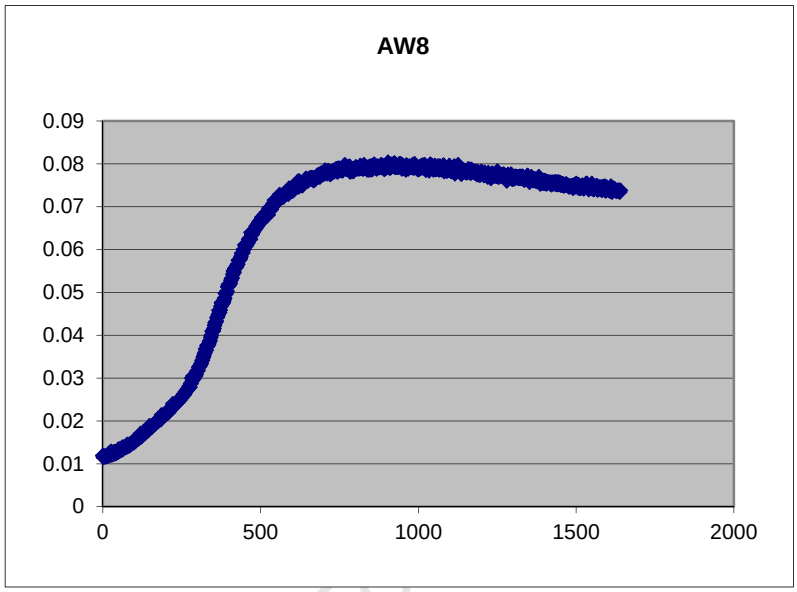
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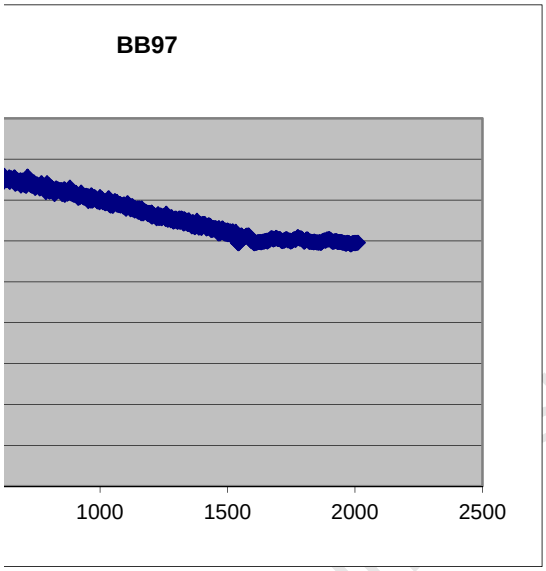
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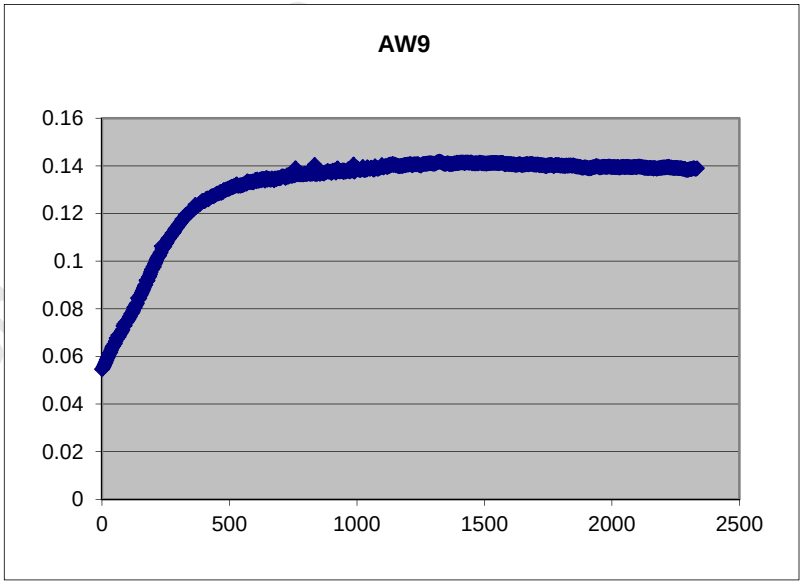
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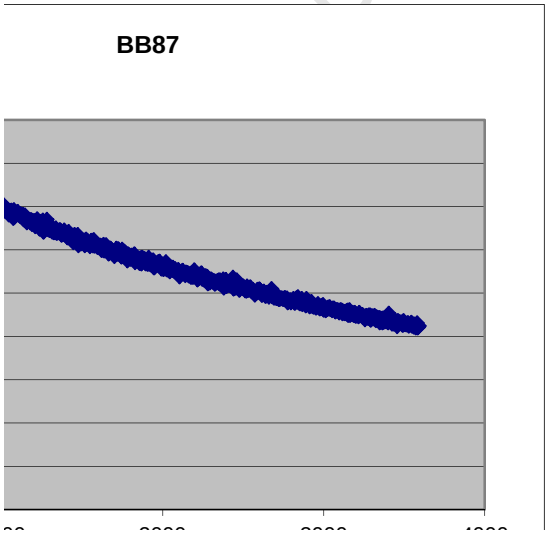
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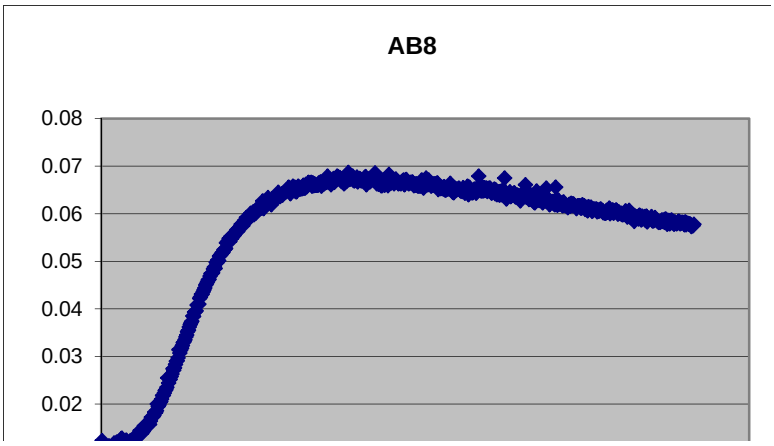
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