University of São Paulo

School of Pharmaceutical Sciences of Ribeirão Preto

Synthesis, antiurolithic activity, and biotransformation studies of galloylquinic acids from *Copaifera* species by filamentous fungi

Síntese, atividade antiurolítica, e estudos de biotransformação de ácidos galoilquínicos de espécies de *Copaifera* por fungos filamentosos

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Area of Specialization: Natural and Synthetic Products
PhD Student: Mohamed Ahmed Mohamed Hamed Abdelsalam
Supervisors: Prof. Jairo Kenupp Bastos
Prof. John Charles Lieske, Department of Internal Medicine, Division of Nephrology and Hypertension, Mayo Clinic School of Medicine, MN, USA

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Summary

SUMMARY

Abdelsalam, Mohamed A. M. H. Synthesis, antiurolithic activity, and biotransformation studies of galloylquinic acids from *Copaifera* species by filamentous fungi. 2018. PhD Thesis. School of Pharmaceutical Sciences of Ribeirão Preto – University of Sao Paulo, Ribeirão Preto, 2018.

Renal stone disease, also known as urolithiasis, is common with a recent overall estimated prevalence rate of 14.8% that appears to be rising, with a five-year recurrence rate of up to 50%. The promising diverse bioactivities of plant extracts rich in galloylquinic acids such as *Copaifera* species leaves prompted our interest to synthesize the tri-substituted 3,4,5-tri-*O*-galloylquinic acid methyl ester (TGAME), with the goal of developing a lead compound for kidney stone prevention. The total synthesis included six steps starting from commercially available quinic and gallic acids. The key step in the synthetic pathway was through Steglich esterification of methyl quinate with 3,4,5-tribenzyloxybenzoic acid using dicyclohexylcarbodiimide and N,N-(dimethylamino) pyridine as the coupling reagents. The chemical structures of the final compound and its synthetic intermediates were elucidated by spectroscopic, spectrometric and spectrophotometric methods of analyses. The potential effect of the compound on calcium oxalate monohydrate (COM) crystal binding to the surface of Madin-Darby Canine Kidney Cells type I (MDCKI) and crystal growth in a *Drosophila melanogaster* Malpighian tubule model were investigated. Membrane, cytosolic and total Annexin A1 (ANXA1), A-enolase and HSP90 amounts were examined by Western blot analysis after subcellular fractionation, then confirmed by immunofluorescence staining of cultured cells. Pretreatment of MDCKI cells with TGAME for up to 6 h significantly diminished COM crystal-binding in a concentration-dependent manner. TGAME (50 μM) significantly inhibited ANXA1 surface expression as evident by immunofluorescence microscopy, whereas intracellular ANXA1 increased. Western blot analysis confirmed ANXA1 expression changes in the membrane and cytosolic fractions of compound-treated cells, whereas the whole cell ANXA1 remained unchanged. TGAME also significantly decreased the size, number, and growth of COM crystals induced in a *Drosophila melanogaster* Malpighian tubule model, and possessed a potent antioxidant activity in a DPPH assay. We also have performed a biotransformation study of galloylquinic acid compounds using filamentous fungi to predict their pharmacokinetic behaviors. The results showed that galloylquinic acids from *Copaifera lucens* leaves (*n*-butanolic fraction, BF) were transformed by *Aspergillus alliaceus* into one major metabolite 3-*O*-methyl gallic acid (M1), which is one of the known metabolites of gallic

acid studied in humans. The biotransformed product was identified by UPLC-DAD-MS/MS and ^{1}H NMR. Pretreatment of MDCKI cells with BF (50 μg/mL) and its transformed product M1 (5 μM) for 3 h significantly diminished COM crystal-binding to these cells. The compounds significantly reduced surface expression of ANXA1 and HSP90 (COM-binding proteins) as evidence by immunofluorescence microscopy, whereas the intracellular level increased. Western blot analysis confirmed these changes in membrane and cytosolic fractions of compound-treated cells, whereas whole cells remained unchanged. M1 also showed a promising antioxidant activity in DPPH assay.

Keywords: Urolithiasis, renal stones, calcium oxalate monohydrate crystals, galloylquinic acids, *Drosophila*, biotransformation, filamentous fungi

RESUMO

Abdelsalam, Mohamed A. M. H. Síntese, atividade antiurolítica, e estudos de biotransformação de ácidos galoilquínicos de espécies de *Copaifera* por fungos filamentosos. 2018. Tese de Doutorado. Faculdade de Ciências Farmacêuticas de Ribeirão Preto – Universidade de São Paulo, Ribeirão Preto, 2018.

Calculo renal, também conhecido como urolitíase, é comum com uma taxa de prevalência estimada global recente de 14,8%, a qual parece estar aumentando, com uma taxa de recorrência em cinco anos de até 50%. As várias atividades biológcas promissoras de extratos de plantas ricas em ácidos galolquínicos, como as folhas das espécies de *Copaifera*, levaram nosso interesse em sintetizar o éster metílico do ácido 3,4,5-tri-*O*-galoilquinico trissubstituído (TGAME), com o objetivo de desenvolver um composto com potencial para prevenção de cálculos renais. A síntese total incluiu seis etapas a partir dos ácidos quínico e gálico disponíveis comercialmente. O passo-chave na via sintética foi a esterificação de Steglich viável do quinato de metila com ácido 3,4,5-tribenziloxibenzóico usando diciclo-hexilcarbodiimida e N, N-(dimetilamino)piridina como reagentes de acoplamento. As estruturas químicas do composto final e seus intermediários sintéticos foram elucidados por métodos espectroscópicos, espectrométricos e espectrofotométricos de análises. O efeito potencial do composto sobre a ligação de cristal monoidratado de oxalato de cálcio (COM) à superfície de células de rim caninas tipo I de Madin-Darby (MDCKI) e o crescimento de cristais em modelo de túbulos Malpighi de *Drosophila melanogaster* foi investigado. As quantidades de membrana, citosólica e total de Annexina A1 (ANXA1), Alfa-enolase e HSP90 foram examinadas por análise de transferência de Western após fracionamento subcelular, as quais foram confirmadas por coloração por imunofluorescência de células cultivadas. O pré-tratamento de células MDCKI com TGAME por até 6 h diminuiu significativamente a ligação de cristal COM de uma maneira dependente da concentração. O TGAME (50 μM) inibiu significativamente a expressão superficial de ANXA1 por microscopia de imunofluorescência, enquanto o ANXA1 intracelular aumentou. A análise de Western Blot confirmou alterações de expressão de ANXA1 na membrana e frações citosólicas de células tratadas com os compostos, enquanto a ANXA1 de células inteiras permaneceu inalterada. O TGAME também diminuiu significativamente o tamanho, o número e o crescimento de cristais de COM induzidos em um modelo de túbulos Malpighi *de Drosophila melanogaster*, o qual apresentou também potente atividade antioxidante em um ensaio de DPPH. Adicionalmente, realizamos estudos de biotransformação de derivados do ácido galoilquínico, utilizando fungos filamentosos, para prever seus

comportamentos farmacocinéticos. Os resultados mostraram que os ácidos galoilquínicos das folhas de *Copaifera lucens* (fração *n*-butanólica, BF) foram transformados por *Aspergillus alliaceus* em um metabólito majoritário, o ácido 3-*O*-metil gálico (M1), que é um dos metabolitos conhecidos do ácido gálico estudado em humanos. O produto biotransformado foi identificado por UPLC-MS/MS. O pré-tratamento de células MDCKI com BF e seu produto transformado por 3 h diminuiu significativamente a ligação de cristal COM a estas células em concentrações de 50 μg/mL e 5 μM, respectivamente. Os compostos reduziram significativamente a expressão superficial das ANXA1 e HSP90 (proteínas de ligação COM) como evidenciado por microscopia de imunofluorescência, enquanto o nível intracelular aumentou. A análise por Western blot confirmou estas alterações nas frações de membrana e citosol das células tratadas com estes compostos, enquanto as células inteiras permaneceram inalteradas. M1 também apresentou atividade antioxidante promissora no ensaio DPPH.

Palavras-chave: Urolitíase, cálculos renais, cristais de oxalato de cálcio monoidratados, ácidos galoilquínicos, *Drosophila*, biotransformação, fungos filamentosos.

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Introduction

1 Introduction

Renal stones (calculi; *s* calculus) disease is considered to be one of the oldest and most ubiquitous diseases known to humankind. Calculi are mineral concretions (deposits) in the renal calyces and pelvis that are found to be free or attached to the renal papillae. By contrast, diffused renal parenchymal calcification is called nephrocalcinosis.

Stones that develop in the urinary tract system are known as urolithiasis or nephrolithiasis, however the later refers specifically to calculi formed inside the kidney. When the stones are formed in the ureters, in this case, it is known as ureterolithiasis, where the stones develop when the urine becomes excessively supersaturated with respect to mineral, leading to crystal nucleation, growth, aggregation and retention with the kidneys (1).

The earliest evidence of this disorder is a bladder stone, dating back to about 4800 BC, found among the pelvic bones in the tomb of a young predynastic Egyptian (2). However, other stones from that era have been reported and out of 9000 mummies examined, only four had positive evidence of calculi, so that the prevalence of the disorder must have been fairly low, at least among upper class Egyptians.

Stones have also been found in North America in the graves of early Indians (ca 1500 BC) (3, 4), but in South America stone disease appears to have been rare amongst the indigenous population until after the Spanish Conquest (5).

In India, references to stone-formation can be located in early Sanskrit documents written between 3000 and 2000 BC (6), and stones were well-recognized in Classical times by Hippocrates in Greece (7) and by Celsus in Rome (6).

Since that time the pattern of stone disease has changed with fluctuation both in the geographical distribution and in the type and composition of stones formed (8, 9). Over the centuries the incidence appears to have been generally increasing, particularly amongst the more industrially developed nations, although there are reports of "troughs" and "waves" in the incidence pattern during and after the two World Wars (8-11).

Over a lifetime, the disease can affect up to 10 -15% of the population (12). After passage of a first stone, the risk of recurrence is 40-50 % within 5 years and 75% within 20 years (13, 14). First-time stone formers do not regularly have a full urine and electrolyte evaluation due to the low incidence of a reversible metabolic cause. However, a reversible metabolic abnormality can be identified in over 90% of recurrent stone formers (12).

The costs associated with stone disease have also risen, increasing from an estimated US$2 billion in 2000 to over US$10 billion in 2006 in the United States alone (15). The

prevalence of stones has been consistently increasing over the past 50 years and further increases are expected owing to changing lifestyle, dietary habits, obesity and global warming (16, 17). Obesity (18), diabetes (18-20), hypertension (17, 20, 21) and metabolic syndrome (22) are considered risk factors for stone formation; conversely, stone formers are at risk of hypertension (21-23), chronic kidney disease (CKD) and end-stage renal disease (ESRD) (23-26).

Urolithiasis of the bladder is a well-documented risk factor for tumor development in humans and rodents and is considered the initiating event that leads to a hyperplastic response, followed by papillomas or diffuse papillomatosis, which may eventually become transitional cell carcinoma (27).

Major advances have been made in the medical and surgical management of patients with kidney stones. Stones can be fragmented using shockwave lithotripsy (SWL) to enable them to pass in the urine, or surgically removed using percutaneous nephrolithotomy (PCNL) or retrograde intrarenal surgery (RIRS). PCNL involves direct endoscopic access into the kidney through an incision in the flank, whereas RIRS is performed using a flexible fibre-optic ureteroscope to access the upper urinary tract through natural passageways. Medical therapies are being used to ease stone passage, promote expulsion and reduce stone recurrence. Important advances have also been made in our understanding of stone pathogenesis.

1.1 Epidemiology

A recent study of epidemiological data from seven countries revealed incidence rates for kidney stones of 114-720 per 100,000 individuals and prevalence rates of 1.7-14.8%, and in nearly all countries, the rates seem to be rising (28). According to data from the National Health and Nutrition Examination Survey (NHANES), the self-reported prevalence of kidney stones in the United States has increased nearly threefold, from 3.2% in the period 1976-1980 to 8.8% in 2007-2010 (16, 29). The lifetime prevalence of kidney stones in the United Kingdom increased by 63% (7.14-11.62%) between 2000 and 2010 (30).

The propensity to form stones varies according to sex, ethnicity and geography. Although historically stones have been two to three times more common in men than in women, recent data indicate that this disparity is diminishing. For example, data from the US Nationwide Inpatient Sample revealed a decline in the male to female ratio for hospital discharges for stones, from 1.7 in 1997 to 1.3 in 2002 (31). The male to female ratio of incident kidney stones also declined in Rochester, Minnesota, USA, from 3.1 to 1.3, between 1970 and 2000 (32). In Florida (USA), a study revealed that the increase in rates in women was greater than that in men

between 1998 and 2004 (33). In Canada, a 48% increase in stone treatment between 1991 and 2010 was primarily accounted for by an increase in procedures among women (34). The reason for the surge in stone disease in women is not precisely understood, but some have proposed that it might be attributable to changes in lifestyle and diet, resulting in increased obesity among women, a known risk factor for stone formation (31).

Racial and ethnic differences in stone prevalence have long been recognized. In the United States, non-Hispanic white individuals have the highest prevalence among racial and ethnic groups (10.3%), followed by Hispanics (6.4%) and non-Hispanic African Americans (4.3%) (16). Comparison of NHANES II (1988-1994) with NHANES III (2007-2010) data has shown that the rise in kidney stone prevalence among Hispanics and African Americans was nearly double that of their white counterparts (16, 29).

Geographical variation in stone disease typically reflects environmental risk factors, with higher stone prevalence in hot, arid climates. In the United States, kidney stones are most prevalent in the south and southeast regions and are lowest in the west of the country (29, 35-37). After controlling for other factors, ambient temperature and sunlight have been shown to be independently associated with stone prevalence (37).

Numerous systemic diseases and factors have been associated with an increased risk of kidney stones. Weight, weight gain, body mass index (18, 38, 39) and diabetes (18, 40) have been shown in large prospective cohort studies to correlate with the risk of incident kidney stones, with a greater effect in women than in men in some cohorts. A multivariable model based on recent NHANES data showed that obesity and diabetes were associated with a 55% and a 59% increased risk of kidney stones, respectively (16). Metabolic syndrome has also been linked to risk of kidney stones, with NHANES data indicating that the number of metabolic traits correlates with the risk of stones (41). Jeong and colleagues (42) detected a 25% higher rate of radiographically detected kidney stones among individuals with metabolic syndrome in a screened population in Asia, after adjusting for confounding variables.

The risk of cardiovascular disease has been associated with a history of kidney stones, although a cause and effect relationship has not been definitively established. Ferraro and colleagues (43) showed a modest increased risk of incident cardiovascular disease among women with a history of stones, but not in men in three large prospective cohorts. Similarly, among individuals registered in the Canadian health care system, a 63% higher risk of incident myocardial infarction was detected among stone formers, with a greater effect in women than

in men (44). A matched pair analysis revealed a 31% higher risk of myocardial infarction in stone formers than in the general population in Olmstead County, Minnesota, USA (45).

1.2 Microstructure of kidney stones

Kidney stones are solid masses, ranging in size from a grain of sand to a pearl (or larger) - a stone does not have to be symptomatic. Depending on their composition, stones are either yellow or brown in color and smooth or jagged in texture or appearance. Globally, approximately 80% of kidney stones are composed of calcium oxalate (CaOx) mixed with calcium phosphate (CaP). Stones composed of uric acid, struvite and cystine are also common and account for approximately 9%, 10% and 1% of stones, respectively (46). They are composed of crystals and a ubiquitous organic matrix, which not only coats the crystals but is also present inside the crystals and the inter-crystalline spaces (47-49). The matrix of calcific stones contains many macromolecules, including osteopontin (which also has a role in bone biomineralization), inter-α-inhibitor (a plasma protein) and urinary prothrombin fragment 1 (UPTF1) - all of which are normally present in the urine (50), albeit in small quantities (50-52). The matrix also contains various forms of lipids, which have been shown to induce crystal nucleation (53, 54). The association between the crystals and the matrix seems to start early upon crystal nucleation and continues throughout the formative and growth phases of the developing stone. Although some urinary molecules, such as UPTF1, are considered crystallization inhibitors, others such as osteopontin can act as both inhibitors and promoters of crystallization (55). These molecules seem to be produced as a protective response against mineralization. However, both CaOx and CaP crystals have been shown to induce the production of macromolecules that inhibit and/or modulate crystallization (52, 56, 57).

1.3 Calculi types

There are four main types of stones and they are named after their major constituents or compositions (Figure 1). Calcium stones are the most common and occur as CaOx and CaP crystals, alone or in combination. Most kidney stones are partially or completely composed of CaOx, which exists as a monohydrate or dihydrate. Individual crystals of CaOx monohydrate (COM) are thin and plate-like, and generally acquire a ‘dumb-bell’ shape through twinning, as seen in urinary sediments. Inside the stones, COM crystals are arranged radially into fan-shaped profiles with distinct concentric laminations, showing outward growth of the crystals and stones. CaOx dihydrate (COD) crystals have characteristic tetragonal bipyramidal envelope shape both in urinary sediment and in kidney stones. CaOx stones are small with shiny exteriors

and generally contain both COM and COD crystals. COM stones are more common than the pure COD stones (58). In mixed stones, COD crystals are predominantly present on the stone surface, which appears jagged. By contrast, pure COM stones have smooth surfaces and they appear as six sided prisms. CaOx stone formation is a multistep process. Hypercalciuria, hyperoxaluria and hypocitraturia are major risk factors.

1.3.1 Calcium Phosphate (CaP) stones

This kind of stones is mainly found as basic CaP (apatite), calcium hydrogen phosphate dihydrate (brushite) or tricalcium phosphate (whitlockite). Pure CaP stones are rare (59). Apatite is the most common crystal in kidney stones and is often a powdery mass that fills the spaces in between other types of crystals, mainly CaOx crystals. Whitlokite is very rare in both kidney stones and urinary sediments. Brushite frequently occurs in kidney stones and is present as rosettes of radially arranged thin blade-like crystals. Hypercalciuria, hypocitraturia and increased urinary pH are major risk factors for CaP stone formation (60).

1.3.2 Uric acid stones

The stones of uric acid comprise 8-10% of all kidney stones worldwide, with a disproportionate prevalence in stone formers who are obese and insulin resistant. Unlike calcium stone types, overly acidic urine (a pH of <5.5) is recognized as the main abnormality responsible for uric acid nephrolithiasis (61). In addition to the insolubility of uric acid at low urinary pH and dehydration, conditions that lead to excessive urinary uric acid excretion, known as hyperuricosuria, have also been associated with uric acid stone formation. These high levels might be due to excess dietary intake of purine-rich foods (62) or endogenous uric acid overproduction, as occurs in conditions such as gout (gouty diathesis). Increased purine catabolism (which can occur in those with myeloproliferative disorders or in those receiving chemotherapy) and the use of drugs that prevent renal reabsorption of uric acid are also contributing factors. Most uric acid stones are compact, appearing like pebbles, with a central core of loosely aggregated anhydrous uric acid crystals surrounded by radiating columnar anhydrous uric acid crystals organized in concentric laminations (58, 63). Some stones display a compact outer layer enclosing a porous friable interior consisting of anhydrous uric acid, uric acid dihydrate and COM crystals mixed with organic material.

1.3.3 Struvite stones

They are large aggregates of orthorhombic ‘coffin-lid’-shaped struvite crystals covered with spherulitic carbonate apatite crystals and mixed with cellular debris, which often included bacteria (63). They are also known as ‘infection stones’, represent 7-8% of all stones worldwide

and are typically caused by increased production of ammonia secondary to infection with urease-producing organisms, such as *Proteus* or *Klebsiella*. The subsequent alkaline urine leads to the formation of magnesium ammonium phosphate hexahydrate crystals (64). Struvite and associated carbonate apatite crystals can grow quickly into large stones referred to as "staghorn calculi", appropriately named for their horn-like projections that occupy the renal pelvis and renal calyces. Although historically feared for their association with high mortality, struvite stones and their association with urosepsis and infections are now treatable with surgical intervention and antibiotics. In the modern era, these stone types are better known for their preponderance for recurrence, particularly in immunocompromised individuals with incomplete stone removal.

1.3.4 Cystine stones

Cystine stones form as result of an autosomal recessive defect in the renal transporter of the amino acid cystine (65). The lack of cystine reabsorption leads to increased urinary cystine excretion. At normal urinary pH, cystine is insoluble and forms cystine crystals that can aggregate to form recurrent kidney and bladder stones. Cystine stones are compact, amber coloured, slightly opaque and with homogenous interiors. Higher magnification of the stone and urinary deposits reveals a unique and characteristic hexagonal structure of the cystine crystals (65).

1.3.5 Iatrogenic stones

Iatrogenic stones can be formed when the urine becomes supersaturated with certain relatively insoluble drugs or their metabolites, leading to crystallization in the renal collecting ducts. For example, patients with HIV who are treated with protease inhibitors such as indinavir and atazanavir are at risk for developing nephrolithiasis (66). Both indinavir and atazanavir are metabolized by the liver, with a considerable proportion of the drug excreted in the urine unchanged, leading to their crystallization and the formation of kidney stones (67). Even when given as part of a multiple drug regimen, atazanavir can crystallize in the urine and form kidney stones (68). Exposure to any one of five different antibiotic classes was associated with nephrolithiasis. Risks were increased 2.3-times, 1.9-times, 1.7-times, 1.7-times, and 1.3-times for sulfas, cephalosporins, fluoroquinolones, nitrofurantoin/methenamine, and broad-spectrum penicillins, respectively, taken 3-12 months before the date of diagnosis.

Poorly soluble dietary contaminants can also crystallize and form stones. For example, melamine has been implicated in the deaths of dogs and cats (69, 70) and caused a major health emergency in China in 2008. Melamine adulteration of infant formula led to the development

of stones and sand-like calculi in the urinary tracts of >294,000 infants (71, 72) >50,000 of whom were hospitalized; six patients died as a result.

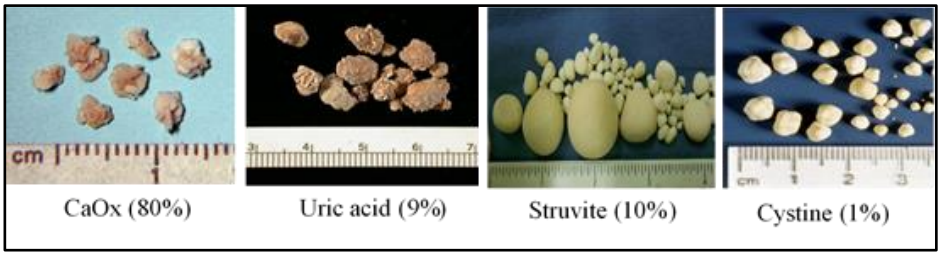

Figure 1. Renal stones types and composition.

1.4 Chemistry and mechanisms/theories of stone formation

Several models of how kidney stone is formed, have been proposed; the two dominating mechanisms for the initiation of stones are commonly described by the terms 'free particle' (in which crystals form 'Randall's plugs' in the tubule) and 'fixed particle' (in which stones grow on so-called Randall's plaques) (Figure 2). Although these models encompass all the possible hypothetical models of how stones begin, no single model can rationalize the evidence observed from all patients with stones-many factors probably contribute. Regardless of the model, the chemical processes of nucleation and crystal growth are essential for the initiation and development of all stone types (73). Stone formation is caused by an abnormal combination of factors that influence the thermodynamic driving force (supersaturation) and the (kinetic rate-controlling) processes involved in the crystallization of the various stone-forming minerals. The principal thermodynamic driving force for both stages is the degree of supersaturation of the fluid within which initiation occurs (73, 74). Whether this takes place intracellularly or extracellularly, the laws of crystallization chemistry should apply.

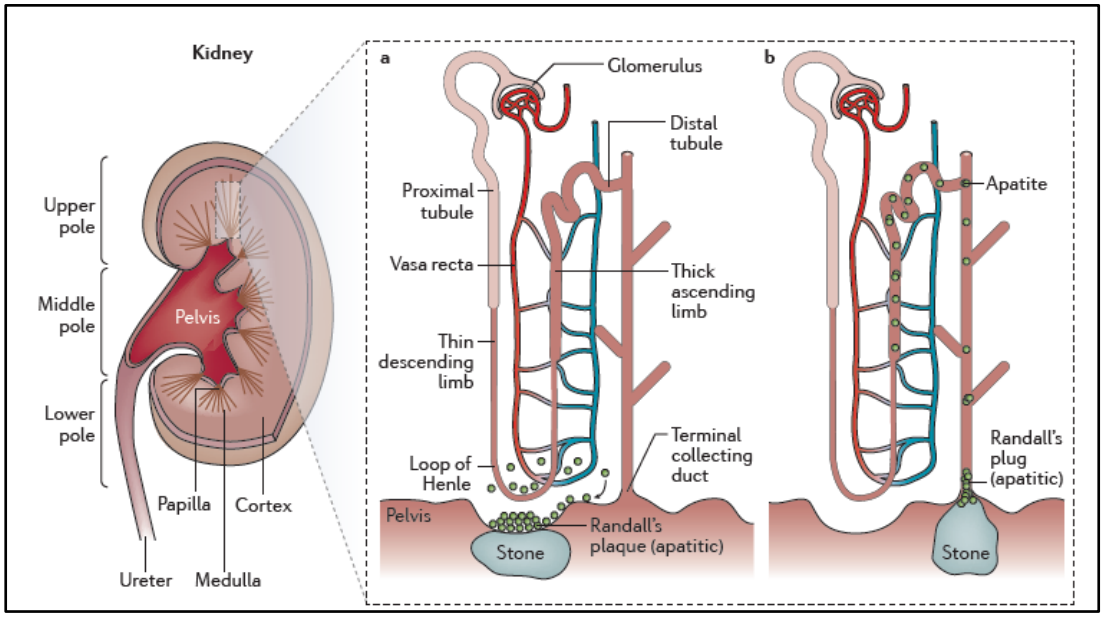

Figure 2. Macroscopic and microscopic morphology of human kidneys and location of stones. (a) According to the fixed-particle mechanism, stones begin as depositions of calcium phosphate (CaP) in the interstitium (apatite), grow outwards reaching the renal papillary surface, become exposed to the pelvic urine and establish a nucleus for the deposition of calcium oxalate (CaOx), leading to the formation of CaOx stones attached to a CaP base, known as Randall's plaques. (b) By contrast, in the free-particle mechanism, for example, CaP, uric acid or cystine crystals form in the renal tubules, move with the urine, aggregate and plug the terminal collecting ducts. These plugs, called Randall's plugs or lesions, are exposed to the pelvic urine. Deposition of CaOx crystals on the CaP plugs leads to the formation of CaOx kidney stones.

1.5 Stages of stone formation

1.5.1 Crystal nucleation

Nucleation (in which solute molecules dispersed in a solvent begin to cluster) is the first stage in crystallization. There are two types of nucleation, either homogeneous or heterogeneous. Homogeneous nucleation requires a high degree of supersaturation with respect to the mineral concerned; *in vitro* this would normally take place in a pure solution containing no particulate matter and in a receptacle that is chemically inert. By contrast, heterogeneous nucleation is the much more likely mechanism through which crystal initiation occurs in the urine (74). The process can occur in the presence of particulate matters consisting of proteins, other organic polymers or crystals of another mineral, and is contained within receptacles lined

with chemically active cell surfaces. Heterogeneous nucleation requires a lower level of supersaturation than homogeneous nucleation for crystal initiation.

1.5.2 Supersaturation of urine with ions

The relative supersaturation (RSS) level at which nucleation occurs is known as the formation product of the mineral concerned; it is not a fixed thermodynamic constant, but covers a range of supersaturation values within which *de novo* crystal nucleation can take place. Its value depends on several factors. First, the length of time of incubation affects the RSS. The longer a given supersaturated solution is left to stand, the more likely it is to precipitate crystals. The higher the initial RSS, the shorter is the nucleation time (75).

1.5.3 Crystal growth and agglomeration

Once a crystal nucleus is initiated inside the kidneys (74), exposure to the urine enables the stone to grow by encrustation (76, 77). As we previously mentioned before, there are two basic pathways (free-particle and fixed-particle mechanisms) for the establishment of a stone nucleus, both of which can be active in any stone former, although stones from idiopathic stone formers are generally formed attached to plaques (78, 79). In the free-particle mechanism (74), crystals nucleate, grow and aggregate within the urine of the renal tubules. Once crystals aggregate to form large particles, they are retained inside the kidneys either by becoming too large to pass through the tubular lumens or by attaching themselves to the tubular (80). In the presence of high supersaturation, crystal deposits occlude the collecting ducts forming Randall's type 2 lesions or plugs (81), which protrude out into the renal pelvis and become exposed to the pelvic urine. Once tubular openings are blocked, stasis can promote the formation of small stones behind the plugs. Similarly, unattached stones in the renal calyces can also form through the free-particle mechanism.

1.6 Prevention, management and current treatment

Although prevention of new calcium stones is possible, there is no effective pharmacological therapy or treatment that can dissolve existing calcium stones. In non-idiopathic calcium nephrolithiasis, the primary conditions should be addressed with specific treatments. In these cases, preventive measures are supportive. In the majority of patients with idiopathic stone disease, behavioral and nutritional interventions are potentially helpful and should be the first step of stone prevention (82). Nutritional advice for patients with calcium stones include increased water intake (>2 liter per day and >3 liter per day in the summer) to provide 24 h diuresis of >2 liter (83), and maintenance of a balanced diet with calcium intake

not <800-1,000 mg per day, reduced meat and poultry intake (≤0.8 g per kg of body weight) (84), reduced salt intake (<2 g per day, which is equivalent to 5 g of table salt (sodium chloride)), avoidance of excess food intake, increased vegetable consumption (85) and avoidance of soda beverages (86). Low calcium diets should be avoided in the majority of patients because they increase intestinal absorption and urinary excretion of oxalate, thereby increasing lithogenesis (87); furthermore, such diets can cause or worsen mineral and bone disorder (MBD) in calcium stone formers. Low oxalate diets are difficult to attain because of the presence of oxalate in many common foods. Only foods with very high oxalate content should be limited or avoided. The concomitant consumption of foods that are rich in oxalate and calcium is a possible strategy to decrease the absorption of oxalate (88). Drug treatment could be considered if stones continue to recur despite the above measures, or if the CKD and/or MBD risks are considerable, or in certain groups of people (for example, flying airline personnel) and in those who have severe urine metabolic abnormalities. For example, thiazides reduce calciuria and might improve bone mineral density (89) and should be considered in patients with high or relatively high urine calcium levels and recurrent calcium stones. However, thiazides have also been shown to decrease stone activity in individuals with normocalciuria (90). Indeed, the American Urological Association guidelines (91) suggest that lowering calciuria with thiazides might be effective regardless of the absolute rate of calcium excretion. Thiazides are appropriate for both CaOx and CaP stones when dietary measures and increased fluid intake have not been successful in preventing stone recurrence. Allopurinol or febuxostat could be useful in patients with calcium stones who are hyperuricosuric (91); the former was shown to be effective in reducing urinary uric acid and stone recurrence in hyperuricosuric CaOx stone formers without other metabolic abnormalities (91). Although no data support its use, the hypouricosuric effect of febuxostat suggests that this drug could be effective in stone formers who cannot tolerate allopurinol (92).

Citrate (generally potassium citrate) use to increase citraturia, which raises the inhibitory activity against calcium crystallization (93), has been shown to be effective in two randomized trials (94). Citrate is indicated in those with recurrent CaOx stones with decreased urinary citrate excretion; in patients with complete or incomplete distal renal tubular acidosis, chronic diarrhoeal states, drug-induced or diet-induced hypocitraturia; and in patients with MBD who form stones. In general, potassium citrate is preferred to sodium citrate because it attenuates calciuria and, therefore, is likely to be more effective in preventing calcium stones (95). However, some concern pertains to overtreatment with citrate, which in theory might

increase the risk of forming new CaP stones because it raises urinary pH (via its metabolism to bicarbonate by the liver). However, in patients with medullary sponge kidney and/or distal renal tubular acidosis and pre-existing high urinary pH, kidney stone recurrence rate is decreased rather than increased after treatment with citrate (96). To prevent frequently occurring uric acid stones, uric acid supersaturation in the urine must be decreased. This can be achieved by increasing urinary volume (>2 liter per day), increasing urinary pH to approximately 7.0, decreasing uricosuria and administering sodium bicarbonate or potassium citrate. Although not supported by data from clinical trials, allopurinol or febuxostat can be used if the patient has hyperuricosuria and dietary measures fail to normalize urinary uric acid (97). Reducing the concentration of cystine in the urine and increasing its solubility will prevent stone formation in this highly recurrent stone disease. The preventive strategy involves increasing water intake to >3 liter per day and the administration of sodium bicarbonate or potassium citrate to raise urinary pH and increase the solubility of cystine. Early in the treatment course, urinary pH should be checked multiple times per day to titrate the quantity of alkali; at a later stage of treatment, the urinary pH should be monitored less frequently. If the previous measures fail to prevent new stones, 6-mercaptopropionyl glycine can be administered, with d-penicillamine as an alternative treatment (88). Given that both drugs can cause proteinuric glomerular diseases, urine should be periodically monitored for proteinuria. Patients with cystine stones require close follow-up because of the high metabolic activity of the disease (with a very high risk of stone recurrence, the rapid growth of these stones, staghorn stone formation, need of surgical procedures and CKD occurrence) and because of the possible adverse effects of treatment.

Treatment of the pain associated with kidney stones (renal colic) is based on the use of NSAIDs as a first choice in the absence of contraindications (98) and, in case of failure in relieving pain, opioids. Intravenous paracetamol (acetaminophen) also seems to be as effective as morphine (99). The use of antispasmodics does not seem to have a significant effect (100). If analgesia cannot be achieved with the previous measures, drainage of the renal pelvis through percutaneous nephrostomy or ureteral stenting and eventually stone removal should be performed.

Surgical interventions such as shockwave lithotripsy, ureteroscopic fragmentation and retrieval and percutaneous nephrolithotomy are common procedures for stone disease. Hydration should be normal and intravenous fluids are only indicated in the case of protracted vomiting because it does not favour stone expulsion but instead increases pain and the risk of complications (renal pelvic rupture and urine extravasation) (101). α-Adrenergic receptor

antagonists (mainly tamsulosin) (102) and calcium channel blockers have been demonstrated to be an effective medical expulsive therapy, believed to be due to their ability to dilate the distal ureter and increase the probability of spontaneous stone passage. The efficacy of these agents in promoting the passage of small distal ureteral stones (<5 mm in size) has recently been decried by two well-designed, randomized, placebo-controlled trials, one of which found efficacy only for larger stones (≥5 mm in size) and the other found no efficacy for stones of any size (103).

Oral dissolution of existing stones is generally effective only with uric acid stones. Two-thirds of these stones can be at least partially dissolved by following the same rules suggested for their prevention: modulating the pH of urine to 7.0, increasing urinary volume and decreasing uricosuria with allopurinol or febuxostat (104).

1.7 Natural products and stone disease

Historically, natural products (secondary metabolites) have been used since ancient times and in folklore for the treatment of many diseases and ailments. Classical natural product chemistry methodologies enabled a vast array of bioactive secondary metabolites from plant, terrestrial and marine sources to be discovered. Many of these natural products have gone on to become current drug candidates. Natural products have been the most successful source of potential drug leads (105-108). They continue to provide unique structural diversity in comparison to standard combinatorial chemistry, which presents opportunities for discovering mainly novel low molecular weight lead compounds. Since less than 10% of the world's biodiversity has been evaluated for potential biological activity, many more useful natural lead compounds await discovery with the challenge being how to access this natural chemical diversity (109).

A number of plants have been studied for their potential use in the modulation and treatment of stone disease. *Copaifera* is a flowering plant that belongs to the family Leguminosae (Fabaceae) Juss., subfamily Caesalpinoideae Kunth (110). The scientific name means "copal-bearer" (or more accurately, copaiba-bearer), since economically important oleo-resin can be obtained from these plants that are used by indigenous Amazonian people for medicinal purposes. They are also important for production of biodiesel and wood, especially *Copaifera langsdorffii*. *Copaifera* consists of 43 species, and is largely distributed in South America (37 species, 28 species in Brazil), Central America (4 species), and Africa (4 species) (111). Several members of the genus are present in Latin America, mainly in the Amazon region (112).

Our group has studied the effect of the *Copaifera langsdorffii* Desf. leaf extract on the ethylene glycol-induced nephrolithiasis in rats, where the plant extract was able to prevent stone formation. Significantly lower oxalate levels and osteopontin (OPN) expression and increased citrate levels were observed after extract administration. Phytochemical analyses showed that the extract is rich in phenolic compounds such as galloylquinic acids that are capable of preventing stone formation (113). In another study, we observed that *C. langsdorffii* have increased levels of magnesium and decreased levels of uric acid in urinary excretions. Treated animals have a significant decrease in the mean number of calculi and a reduction in calculi mass. Calculi taken from extract treated animals were more brittle and fragile than calculi from untreated animals. Moreover, breaking calculi from untreated animals required twice the amount of pressure as calculi from treated animals (114). The anti-inflammatory and antiurolithic effects of polyphenolic compounds from *Quercus gilva* Blume were investigated in a previous study, where some isolated compounds from the plant showed potent anti-oxidative and anti-inflammatory activities. These compounds were further tested before for their inhibition of the gene expression of the inflammatory cytokines, where some other phenolic compounds in the extract showed dose-dependent inhibitory activities on gene expression of COX-2 and IL-1 (115).

Recently, epigallocatechin gallate (EGCG), a green tea polyphenol was reported to inhibit free-radical production induced by oxalate (116). It also decreases binding of calcium oxalate monohydrate crystals onto renal tubular cells via decreased surface expression of α-enolase (117).

PGG (1,2,3,4,6-penta-*O*-galloyl-beta-D-glucose), a polyphenolic and water soluble gallotannin (118) isolated from gallnut of *Rhus chinensis* MILL, is known to have several biological effects towards stone disease, including attenuation of renal cell migration, hyaluronan expression, and crystal adhesion (119). The compound also reduces renal crystallization and oxidative stress in a hyperoxaluric rat model (120).

Natural products including gallotannins found also in green teas have been studied as potentially novel treatments to prevent crystal retention and kidney stone formation. Gallotannin significantly inhibited COM crystal growth and binding to Madin-Darby Canine Kidney Cells type I (MDCKI) renal epithelial cells. It significantly attenuated oxalate-induced mRNA and protein expressions of monocyte chemoattractant protein 1 (MCP-1), OPN, nicotinamide adenine dinucleotide phosphate (NADPH) oxidase subunit p22phox and p47phox in human primary renal epithelial cells (HRCs). The molecule also reduced the levels of reactive

oxygen species (ROS) and malondialdehyde (MDA) as well as enhanced antioxidant enzyme superoxide dismutase (SOD) activity in oxalate treated HRCs (121).

Phyllanthus niruri and *Costus arabicus* L. (*C. arabicus*) are plants used in Brazilian folk medicine to treat urolithiasis. The Aqueous extract of the later inhibits calcium oxalate crystal growth and adhesion to renal epithelial cells (122). *P. niruri* is a plant belonging to the Euphorbiaceae family, which have a worldwide distribution. It is used in Brazil by patients with urolithiasis (123, 124). It was reported that the oral administration of *P. niruri* aqueous extract to rats induces an inhibitory effect on vesical CaOx crystal growth, which is associated with a reduction in the urinary excretion of glycosaminoglycans (GAGs) and with an increase in the content of these macromolecules in the calculi compared with untreated animals (125). Also, *P. niruri* significantly reduces the endocytosis of CaOx crystals in MDCK cells in culture (126). The effect of the extract of *P. niruri* on crystal deposition in experimental urolithiasis was also studied before (127). Moreover, it has an inhibitory effect on crystal growth, which is independent of citrate and Mg, but might be related to the higher incorporation of GAGs into the calculi (125).

Holarrhena antidysenterica, a plant that has a traditional use in the treatment of stone disease, where its crude extract possesses antiurolithic activity, possibly mediated through the inhibition of CaOx crystal aggregation, displaying antioxidant and renal epithelial cell protective activities (128).

In a previous study, the *in vitro* effect of lemon and orange juices on calcium oxalate crystallization has been investigated, where lemon juice was found to inhibit the rate of crystal nucleation and aggregation (128).

1.8 Herbs for prevention of urolithiasis (129)

Barberry root bark (*Berberis vulgaris*) - Barberry was found to inhibit calcium oxalate crystallization and prevent kidney damage caused by oxidative stress in animal studies. The water extract was the most effective preparation (130). **Dose:** Tea: 1 tsp. dried root bark to 300 mL water, decoct for 10-15 minutes, steep for 30 min, taken as 120 mL BID/TID.

Black cumin seed (*Nigella sativa*) - in animal studies, the use of this herb significantly protected against experimentally induced formation of CaOx stones (131). **Dose:** Tea: ½ tsp. dried seed, 240 mL hot water, steep covered 20 minutes, take 120 mL BID/TID.

***Chanca Piedra*/Stonebreaker** (*Phyllanthus niruri*) - is native to tropical regions and has a long history of use for helping to prevent and pass kidney stones. In several *in vitro* and animal studies, daily intake of this herb helped to prevent the formation of kidney stones (132).

In a human study, this herb was found to reduce urinary calcium levels in patients with hypercalciuria (132). It also slowed the growth of existing stones (127). **Dose:** Tea: 1-2 tsp. dried herb, 240 mL hot water, steep 30 min. Take 2-3 cups per day. Tincture (1:5): 3-6 mL (60-120 gtt.) TID.

Evening primrose seed oil (*Oenothera biennis*) **-** in a human study, daily ingestion of the oil (1000 mg per day) significantly increased citraturia (urinary citrate levels) while reducing urinary oxalate, calcium and the Tiselius risk index, which is a measurement of risk for forming kidney stones (133). **Dose:** Tea: 2 tsp. dried leaf, 240 mL hot water, steep for 45 min, taken as 240 mL BID, Tincture (1:5): 1.5-3 mL TID.

Fagolitas - is a Spanish herbal formula containing fluid extracts of Uva Ursi, Corn Silk, *Ricinus zanzibarensis*, tincture of Saw Palmetto, mother tincture of Buchu, glycerin and Anise essence. Animals given this formula had a significant reduction of papillary and intratubular calcification in the kidneys (134).

Fenugreek seed (*Trigonella foenum-graecum*) **-** the seeds of this herb are commonly used in northern Africa to prevent and treat kidney stones. In an animal study, it was found that Fenugreek seed significantly reduced calcification in the kidney and helped in preventing kidney stones (135, 136). **Dose:** Tea: 1-2 tsp. dried seed, 300 mL water, decoct for 15-20 minutes, steep 30 min, taken as 120-180 mL TID, Tincture (1:5): 2-4 mL (40-80 gtt.) TID.

Gokshura fruit/root (*Tribulus terrestris*) **-** this herb is an Ayurvedic rasayana, nephroprotective agent, and is commonly used in India and China to treat urinary tract disease. In animal studies, it prevented the formation of kidney stones and helped to reverse early stage urolithiasis. *In vitro* research supports the animal data and further suggest that this herb also protects against calcium oxalate-induced renal injury (137). **Dose:** Powder: 1 tsp. TID.

Hibiscus flowers (*Hibiscus sabdariffa*) **-** in animal studies, Hibiscus was able to increase urinary oxalate excretion and it significantly reduced oxalate deposition in kidneys (138). In another study, patients with previous history of kidney stones, Hibiscus tea (2 cups per day) increased oxalate and uric acid excretion and enhanced urinary citrate levels (139). **Dose:** Tea: 1-2 tsp. dried flowers, 240 mL hot water, steep for 20 min, taken as 240 mL BID/TID, Tincture (1:2 or 1:5): 2-4 mL TID.

Jin Qian Cao herb (*Desmodium styracifolium***) -** this Chinese herb inhibits urinary calcium excretion and increases urinary citrate, significantly reduces formation of renal stones (140). **Dose:** Tea: 2-3 tsp. dried herb, 240 mL hot water, steep 40 min. Taken as 2-3 cups per day.

Rose hips (*Rosa canina*) - experimental animals were given an infusion of Rose hips, Rose hips and magnesium, or magnesium alone. Both the herb and the mineral promoted an increase in urinary citrate and reduced urinary calcium excretion (141). **Dose:** Tea: 1 tsp. Rose hips, 240 mL hot water, steep for an hour. Taken as 120 mL TID.

Rupture wort herb (*Herniaria hirsuta*) - in animal studies, this herb inhibited the deposition of CaOx crystals in kidneys (142). **Dose:** Tea: 1 tsp. dried herb, 240 mL water, decoct 5-10 min, taken 1-2 cups per day.

Shatavari root (*Asparagus racemosus*) - this important Ayurvedic Rasayana (rejuvenative remedy) was found to inhibit formation of CaOx stones in animal study (143). **Dose:** Tea: 1 tsp. dried, powdered root, 240 mL water, decoct 10 min, steep for 40 min, taken as 2 cups/day. Tincture (1:5): 2-4 mL (40-80 gtt.) TID.

Varuna bark (*Crataeva nurvala*) - daily intake of this Ayurvedic herb reduced urinary calcium excretion and kidney stone formation (144). **Dose:** Tea: 2 tsp. dried bark, 350 mL water, decoct 15 min, steep for an hour. Taken as 240 mL 2-3 times per Day Tincture (1:5): 4-5 mL (80-100 gtt.) TID.

Water plantain root (*Alisma orientalis*) - the Chinese herb Ze Xie/Water Plantain root has a long history of use in traditional and complimentary medicine (TCM) for treating dysuria, edema, and cystitis. In animal studies, it was also able to inhibit experimentally induced calcium urolithiasis (145). **Dose:** 2 tsp. dried root, 300 mL water, decoct 20 min, steep for an hour, taken as 120 mL TID.

Wu Ling San - this TCM formula is comprised of aqueous lantain root (*Alisma orientalis*), *Polyporus umbellatus*, *Atractylodes macrocephala*, Fu Ling (*Wolfiporia cocos*), and Cinnamon bark. In animal studies it effectively reduced CaOx deposition in rat kidneys (146). **Dose:** Powder: 6-9 grams BID, Tablets: 4-5 tablets BID.

Herbs for treating kidney stones

Many herbs in TCM, Ayurveda, Native American medicine, Eclectic/Physiomedical medicine and European traditions have a long history of being used to help with kidney stones and urinary calculi, among them:

Couch grass rhizome (*Elymus repens*) - is a soothing diuretic that can be useful as part of a formula to make passing stones easier. It also promotes uric acid excretion. Therefore, it can help to prevent uric acid stones. **Dose:** Tea: 2-3 tsp. dried rhizome, 12 oz. water, decoct 30 min, steep for 30 min, taken as 1 cup 3 times/day Tincture: (1:4 or 1:5, 1:2.5): 3-5 mL (60-100 gtt) TID/QID.

Golden rod herb (*Solidago spp.*) - herbalists in the UK often use Solidago with Pellitory-of-the-Wall or Parsley Piert for helping to pass kidney stones. The British herbalist Christopher Hedley, AHG, says that he has seen this simple formula "cause stones to vanish". The patients never noticed the stone passing and upon a follow up with an ultrasound, they had disappeared. **Dose:** Tea : 1-2 tsp. dried herb, 240 mL hot water, steep covered, 20-30 min, take 2 cups/day Tincture (1:5): 2-3 mL (40-60 gtt.) TID/QID.

Horse Chestnut seed (*Aesculus hippocastanum*) - the specific indications for *Aesculus* are for throbbing pain with edema and inflammation. It is most often used for hemorrhoids, varicose veins and trauma injuries. The analgesic and anti-inflammatory effects also help with the intensive pain caused by kidney stones and reduce swelling of the ureter, thus allowing stones to pass more easily. **Dose:** Tincture (1:2): .25-.75 mL (5-15 gtt.) TID, Capsules: A standardized product (16-20% Escin) has been used in several studies with a dose of 300 mg of the extract every 12 h.

Horsetail herb (*Equisetum arvense*) - this herb is rich in silicic acid and helps strengthen bones, teeth, hair, skin and nails. It also helps speed healing of minor kidney damage and hematuria caused by passing stones. In the UK, Horsetail has the reputation of promoting expulsion urinary calculi. **Dose:** Tea: 1 tsp. dried herb, 240 mL water, decoct 15 min, steep 1 h, taken as 120 mL 3times/day, Tincture (1:5): 1-2 mL (20-40 gtt.) TID.

Hydrangea root bark (*Hydrangea arborescens*) is a native American shrub which is one of the most effective urinary tract analgesics. It is indicated for genito-urinary tract pain and spasm and it is used with Khella, Lobelia, Kava, Horse Chestnut, and Yucca root for acute pain caused by kidney stones. **Dose:** Tea: 1 tsp. dried bark, 240 mL cold water, steep for 1 h. Taken as 120 mL TID Tincture (1:5): 2-3 mL TID.

Jin Qian Cao herbs (*Desmodium styracifolium*) - there are three herbs known as Jin Qian Cao. Out of the three, Desmodium and *Glechoma longituba* are believed to be more effective for helping to pass kidney stones. Lysmachia (also known as Jin Qian Cao) is believed by some practitioners to be more useful for treating gallstones, but it is also commonly used in formulas for helping to pass kidney stones. **Dose:** Tea: 2-3 tsp. dried herb, 240 mL hot water, steep 40 min. Taken as 2-4 cups per day.

Kava root (*Piper methysticum*) – it was introduced to western medical practice by the British explorer Captain Cook. In the U.S.A, the Eclectic physicians primarily used it for urinary tract pain. It helps relax the ureters, allowing stones to pass more easily and diminishes colicky, spasmodic pain. **Dose:** Tea (Decoction): 1-2 tsp. dried root, 240 mL water, decoct 15 min, steep

for 1 h, then blend. Taken as 120 mL QID, Tincture (1:4, 1:5): 2-4 mL (40-80 gtt.) TID/ QID, Capsules: Standardized (60 mg Kava lactones), 2-4/day.

Khella seed (*Ammi visnaga*) – this is a northern Africa plant is an effective antispasmodic, useful for relieving spasm and pain in the urinary tract, gall bladder, respiratory tract and cardiovascular system. Khella is very useful as part of a protocol for helping to pass urinary calculi. **Dose:** Tea: 1 tsp. dried seeds, 240 mL hot water, steep covered for 30 min, taken as 120 mL TID, Tincture (1:5): 1-2 mL TID.

Lobelia seed/fresh herb (*Lobelia inflata*) is primarily known as a respiratory remedy used for asthma and spasmodic coughs. It is also an effective antispasmodic for the cardiovascular, genito-urinary and musculoskeletal systems. The tincture of lobelia seed or the tincture of the green flowering herb is highly useful for relieving acute pain caused by stones passing through the ureters. It should be used in formulas combined with Khella, Hydrangea, or Horse Chestnut. **Dose:** Tincture: fresh herb (1:2), 0.5-1 mL (10-20 gtt) TID/QID, seed (1:5), 0.25-0.75 mL (5-15 gtt) TID/QID.

Marshmallow root (*Althea officinalis*) is the most soothing and mucilaginous herbal diuretic. Consuming enough quantities of the tea can help ease passage of urinary stones and relieve inflammation and tissue damage. **Dose:** Tea: 1-2 tsp. dried herb, 240 mL hot water, steep covered 20 min, take 120-240 mL TID.

Pellitory of the Wall herb (*Parietaria diffusa*) is used in the UK as a diuretic, kidney trophorestorative and to help pass urinary calculi and stones. It is often combined with Goldenrod, Parsley or Parsley Piert to help prevent stones or assist in their passage. **Dose:** Tea: 1-2 tsp. dried herb, 240 mL hot water, steep 30 min, take 120 mL TID, Tincture (1:5): 1.5-2 mL (30-40 gtt.) QID.

Punarnava herb (*Boerhaavia diffusa*) is a common Indian weed is used as a kidney restorative and to help expel kidney stones. In an *in vitro* study it was able to inhibit formation of struvite stones, but there is no data about this effect *in vivo*. **Dose:** Powder: 1 tsp. TID.

Varuna bark (*Crateava nurvala*) is an Ayurvedic herb is used to help prevent kidney stones and is also used with banana stem (*Muse paradisiaca*) for treating kidney stones. In a recent human study. The authors state that this formula "helped to dissolve renal calculi, facilitated their passage and reduced pain." **Dose:** Tea: 2 tsp. dried bark, 350 mL water, decoct 15 minutes, steep for 1 h. Taken as 240 mL 2-3 times per day, Tincture (1:5): 4-5 mL (80-100 gtt.) TID.

Wild Carrot seed (*Daucus carota*) **-** British herbalist Anne McIntyre FNIMH used Wild Carrot seed along with Parsley Piert (Alchemilla arvensis) for helping to expel kidney stones. **Dose:** Tincture (1:5): 5 mL TID - 2.5 mL (50 gtt) of each.

Yucca root (*Yucca spp.*) - Alabama herbalist Phyllis Light, RH (AHG) uses Yucca root to help ease passage of kidney stones and relieve urinary tract pain. **Dose:** Tea: 1 tsp. dried root, 300 mL water, decoct 15 min, steep for 20 min, take 120 mL TID, Tincture: 1-2 mL (20-40 gtt.) TID.

Objectives

2 Objectives of the PhD project

2.1 General objectives

- Investigating the biological activity of galloylquinic acid compounds towards renal stone disease (urolithiasis) for the pre-clinical discovery of new therapeutic candidates as antiurolithic agents.

2.2 Specific objectives

- The total synthesis of a bioactive galloylquinic acid compound (3,4,5-tri-*O*-galloylquinic acid methyl ester, TGAME).
- Characterization of the obtained synthetic compound and its chemical intermediates by spectroscopic and spectrophotometric techniques that include: HPLC-UV, LC/MS-MS, ^{1}H NMR, ^{13}C NMR, two dimensional (2D) NMR and IR.
- Biotransformation studies of galloylquinic acid compounds from *Copaifera lucens*, by filamentous fungi, aiming to predict the pharmacokinetic profile of these compounds.
- Antiurolithic activity of TGAME, galloylquinic acid compounds and their biotransformed metabolites by performing the following assays:
  - Cytotoxicity study in Madin-Darby Canine Kidney Cells type I (MDCKI) and human renal cells (JL).
  - Cell-calcium oxalate monohydrate (COM) crystal adhesion assay.
  - Subcellular localization of potential crystals receptors [α-enolase (enolase-1), Annexin A1 and HSP90] by protein extraction followed by Western blot analysis.
  - Neutralization of a potential COM-binding proteins using a specific antibody.
  - Confocal microscopy and immunofluorescence staining.
  - The use of *Drosophila melanogaster* (fruit fly) for studying calcium oxalate crystals growth, size and number by the inhibitory effects of the synthetic compound.

Conclusion

3 Conclusion

In summary, we report that the plant 3,4,5-tri-*O*-galloylquinic acid methyl ester (TGAME, 6) inhibited COM crystal adhesion to renal cells and this effect is mediated by decreased expression of ANXA1 on cell surface. Thus, cell surface expression of ANXA1 might be a key pathogenic factor in crystal retention and urinary stone formation *in vivo*. Both M1 metabolite and BF as well as compound 16 also inhibited COM crystals binding to MDCKI cells with two different mechanisms of actions.

TGAME also significantly decreased CaOx crystal number, size and total crystal area within MTs of *Drosophila* models, as well as showing a potential antioxidant activity by free radicals scavenging capability. Our findings may also be relevant for the observed decrease of crystal deposition in urolithiasis animal models-treated with *Copaifera* leaf extracts. In addition to, our findings support a promising role for TGAME in the prevention and modulation of new or recurrent renal stone formation. Moreover, our results support the hypothesis of Verkoelen and Verhulst that crystal binding is preceded by pathologic alterations in cell surface binding molecules, therefore further preclinical and clinical studies should be performed for the use of this compound in urolithiasis.

We also reported that galloylquinic acids from *Copaifera lucens* leaves (*n*-butanolic fraction, BF) were all transformed by *Aspergillus alliaceus* into one major metabolite 3-*O*-methyl gallic acid (M1), which is one of the known metabolites of gallic acid studied in humans. These data can provide a tool for predicting the metabolic profile of galloylquinic acids or related compounds *in vivo*.

Both BF and its transformed product (M1) significantly diminished COM crystal-binding to MDCKI cells in concentrations of 50 μg/mL and 5 μM, respectively. The compounds also exhibited antioxidant activities.

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