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Evaluation of the anti-urolithiatic potential of *Punica granatum* extracts

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List of abbreviations

3D: Three dimensions

AP-1: Activated protein-1

BC: Before Christ

BCE: Before Christ exited

BK: Bikunin

BMP: Bone morphogenetic protein

BSP: Bone sialoprotein

Ca^{2+}: Calcium ion

CaOX: Calcium oxalate

CaP: Calcium phosphate

CASA: Computer-assisted sperm analysis

COD: Calcium oxalate dihydrate

COM: Calcium oxalate monohydrate

COT: Calcium oxalate trihydrate

CUTI: Chronic urinary tract infection

Cyt-C: Cytochrome C

DNA: Deoxyribonucleic acid

EAU: European association of urology

EC: Enzyme commission

EG: Ethylene glycol

Eq: Equivalent

Fe^{2+}: Ferrous cation

Fe^{3+}: Ferric cation

FRAP: The Ferric Reducing Antioxidant Power

GSH: Glutathione

HCl: Hydrogene chloride

HK-2: Human renal tubular epithelial

HPRT: Hypoxanthine-guanine phosphoribosyltransferase

IC50: Half maximal inhibitory concentration

KS: Kidney stones

Lyso-PC: Lysophosphatidylcholine

MCP-1: Monocyte chemoattractant protein-1

MDA: Malondialdehyde

MDCK: Madin-Darby Canine Kidney

Mg^{2+}: Magnesium ions

$MgNH_4PO_4 \cdot 6H_2O$: Magnesium ammonium phosphate stones

MGP: Matrix gla protein

NADPH: Nicotinamide adenine dinucleotide phosphate

NaOH: Sodium hydroxide

NFκB: Nuclear factor κB

NH^{4+}: Ammonium

NRK-52E: Normal rat kidney epithelial-like

N-Smase: Neutral sphingomyelinase

OD: Optical density

OPN: Osteopontin

P38-MAPK,JNK: P38 Mitogen-activated protein kinase.

PDB: Protein Data Bank

PGF: *Punica granatum* flower

PGPd: *Punica granatum* delipidated

PGPnd: *Punica granatum* non delipidated

PLA-2: Phospholipase A2

PO_4^{3-}: Phosphate ion

PUFA: Polyunsaturated fatty acids

RBC: Red blood cells

ROS: Reactive oxygen species

Rpm: Rotations par minute

RTECs: Renal tubular epithelial cells

RUNX-2: Runt-related transcription factor-2

S.C.A: Sperm Class Analyzer

SD: Standard deviation

SEM: Standard error of the mean

TE: Trolox equivalent

TEAC: Trolox equivalent antioxidant capacity

TPTZ: Tris(2-pyridyl)-s-triazine

VAP: Average path velocity

VCL: Curvilinear velocity

VSL: straight-line velocity

XO: Xanthine oxidase

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Introduction

Introduction

Kidneys are complex organs within the renal-urologic system that are essential for maintaining the vitality of the human body. They act as a filtering system, excreting out all waste substances and fluids, thus creating a stable state for cell and tissue metabolism through the maintenance of homeostasis with the electrolyte and acid-base balances in the bloodstream **(Sohgaura & Bigoniya, 2017; Chalmers, 2019)**.

Morphological and/or functional impairment induces the establishment of several renal diseases, like kidney stone (KS) disease also known as urolithiasis. According to recent epidemiologic data, KS is common and a major public health concern; it prevalence keeps on increasing, especially the formation of calcium oxalate monohydrate crystals (COM) **(Stamatelou & Goldfarb, 2023**). Various factors contribute to the development of this disease, ranging from external factors such as climate and dietary habits to internal factors as oxidative stress and purine metabolism **(Devi et *al*., 2023)**.

Surgical interventions are usually implemented for the management of this disease **(Geraghty et *al*., 2017)**; however, these procedures are costly and can result in acute kidney injury and a decline in kidney function, and their recurrence rate is very high. Despite the effort to use pharmaceutical treatments such as thiazide diuretics and alkaline citrate in an attempt to manage urolithiasis, however their effectiveness in comparison to their side effects remains unconvincing **(Bashir and Gilani, 2011; Sikarwar et *al*., 2017; kachkoul et *al*.,2023)**. The traditional medicine used pomegranate or *Punica granatum* plant for centuries in the treatment of various diseases, including kidney diseases **(Jouad et *al*., 2001; Kachkoul et *al*., 2023)**. To confirm their beneficial effect from a scientific viewpoint, researchers are currently conducting studies to examine their effects on several models, including *in vivo, in vitro,* and semi-*in vivo* investigations. Furthermore, bioinformatics-based *in silico* techniques are currently employed to discover novel compounds for the treatment of this ailment, aiming to minimize adverse reactions **(Devi et *al*., 2023)**.

Our study aims to investigate the anti-urolithiasis activity of *Punica granatum* extracts via different study models. *In vitro* via enzyme inhibitory activities, *in silico* implementing molecular docking and semi-in *vivo* via the use of spermatozoa cells. To date spermatozoa have never been used as a model in urolithiasis activity.

Literature review

I.1. Kidney anatomy and physiology

I.1. 1. Anatomy

Kidneys are paired, bean-shaped organs located retroperitoneally on either side of the spinal column, the lower pole of the kidney is just about the third lumbar vertebrae, while the upper pole is near the 12th thoracic vertebrae. Due to the presence of the liver on the right side, the right kidney lies slightly lower than the left one. Usually, the concave surface of the kidneys is oriented facing the spine. However, specific aberrations could result in different kidney orientations without affecting the kidney's overall function. On average, the kidney is about 12 cm long and weighs roughly about 150g **(Sohgaura & Bigoniya, 2017;Chalmers, 2019)**. Three different layers surround kidneys**:**

- **Renal fasci:** also known as fibrous capsule is the outmost layer of thin connective tissue, that fixes the kidney to surrounding tissue, including the abdominal wall, serving as structural support;
- **Adipose capsule:** usually described as a fat cushion that protects the kidney from injury and helps maintain a stable position within the abdominal cavity;
- **Renal capsule:** representing the innermost layer of connective tissue that acts as a support system, by maintaining the kidney's structure and shape and protecting its internal tissue (**Taylor, 2023)**.

The internal kidney structure is as illustrated in **figure 01**.

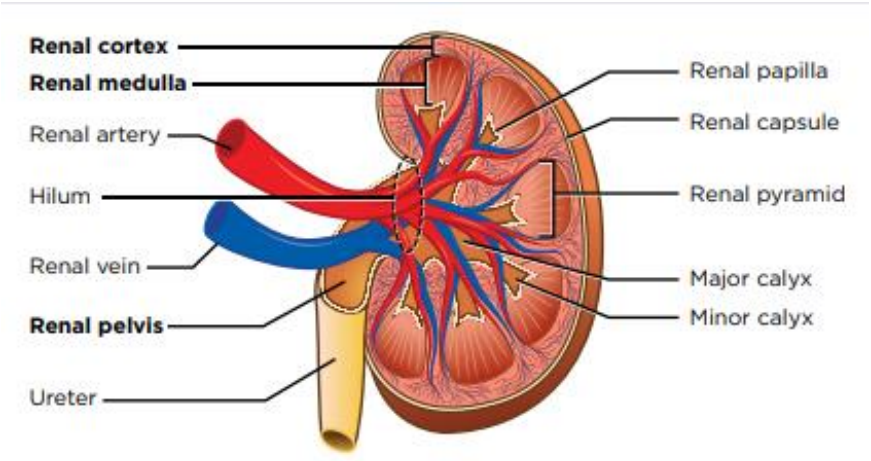

Figure 01: Kidney general anatomy **(Taylor, 2023)**

I.1. 2. Physiology

The kidneys have several functionalities, their main function is the regulation and maintenance of the composition and volume of body fluids, it's a filtering system that processes blood plasma and removes unwanted substances such as urea, uric acid, toxins, excess hormones, water, and electrolytes through the urine via glomerular filtration and tubular reabsorption and secretion. Moreover, it contributes to some metabolic pathways such as the activation of vitamin D, production of renin and erythropoietin **(Sohgaura & Bigoniya, 2017;Chalmers, 2019)**.

Any alteration in the physiology and function of the kidney will lead to the appearance of kidney disease which can develop into chronic kidney disease (CKD), including urolithiasis **(Stamatelou & Goldfarb, 2023)**.

I.2. Pathophysiology of nephrolithiasis

I.2. 1. Definition

Urolithiasis or nephrolithiasis comes from the Greek words *Uro*, which means urinary, *nephros*, which means kidney, and *lithos*, which means stone. Kidney stone or urinary stone disease affected Men even before the urinary tract was identified; it was first mentioned in ancient medical texts in Asûtu of Mesopotamia between 3200 and 1200 BC **(Shah & Whitfield, 2002; Stamatelou & Goldfarb, 2023)**.

Currently kidney stone, is considered the most common urological disorder worldwide **(Stamatelou et *al*., 2003; Romero et *al*., 2010; Stamatelou & Goldfarb, 2023)**. In literature these stones are described as crystal aggregates entailing the deposition of inorganic substances along with organic matrix within the pelvicalyceal system or renal parenchyma through a complex step by step process **(Khan & Hackett, 1993; Khan et *al*., 2016; Peerapen & Thongboonkerd, 2023).**

I.2. 2. Formation of nephrolithiasis

As previously mentioned, nephrolithiasis, consist of crystal aggregates; the exact details behind the formation of these crystallin stones within the body are not fully understood **(Paliouras et *al*., 2012; Tavasoli & Taheri, 2019).**

However, based on various evidence from human renal tissue biopsies and intra-operative endourologic imaging, several hypothesis have been proposed in an attempt to explain the initiation and formation of these stones major ones being free particle theory, fixed particle theory, and Randall's plaque hypothesis **(Paliouras et *al*., 2012; Tavasoli & Taheri, 2019).**

According to the different hypotheses proposed current literature suggests that regardless of their type, the formation of nephrolithiasis includes a complex cascade of events influenced by numerous biological factors (promotors, inhibitors), referred to as "lithogenesis". This process initiates with urinary supersaturation, then crystal nucleation, growth, aggregation, and eventually retention within the kidney. Yet these conclusions raise controversial data, as this mechanism can occur even in non-stone formers as elucidated in **Figure 02.** However, the size of the crystals formed in healthy individuals is small enough (≤20 µm) to pass through the urinary tract without interacting with the epithelial cells **(Moe, 2006; Aggarwal et *al*., 2013; Espinosa-Ortiz et *al*., 2019; Tamborino et *al*., 2024)**.

At a certain level of supersaturation due to absence of crystallization inhibitors or a reduced urinary volume, solute particles start to combine leading to the formation of loose clusters through nucleation process, which can occur homogeneously or heterogeneously.**(Khan, 1997; Miller et *al*., 2007; Kachkoul et *al*., 2023)**. Once the initial nucleus is formed, additional crystal compounds build up as the crystal increase in size causing crystal growth **(Fleisch, 1978 ;Aggarwal et *al*., 2013; Espinosa-Ortiz et *al*., 2019; Devi et *al*., 2023)**. Then, crystal particles collision together to form aggregates, through the aggregation process creating crystal stones big enough to be retained within the urinary tract **(Fleisch, 1978;Aggarwal et *al*., 2013; Espinosa-Ortiz et *al*., 2019; Devi et *al*., 2023)**.

I.3. Type of stones and their risk factors

I.3.1. Risk factors

Urinary risk factors of kidney stone formation have been shown to associate with many risk factors including the intrinsic factors (age, gender, family history, genetic factors, race, microbiome, systemic diseases, urinary composition..etc) and extrinsic factors such climate, geography, occupation, diet and ..etc **(Stamatelou & Goldfarb, 2023).**

Despite that, the stone formation mechanism follows the same cascade of events for all stone types. However, morpho-constitutional analysis reveals that the chemical composition of a stone is different from another's, as are the stone's crystalline form and structural characteristics **(Daudon et *al.*, 1993; Courbebaisse, 2016; Kachkoul et *al.*, 2023)**. Literature reports that there are six basic morpho-constitutional categories, and each one could be further split into subtypes according to various factors involved in their formation **(Daudon et *al.*, 2016; Kachkoul et *al.*, 2023)**. Still, as reviewed by many studies, the most common stone categories are as elucidated in **Figure 02**.

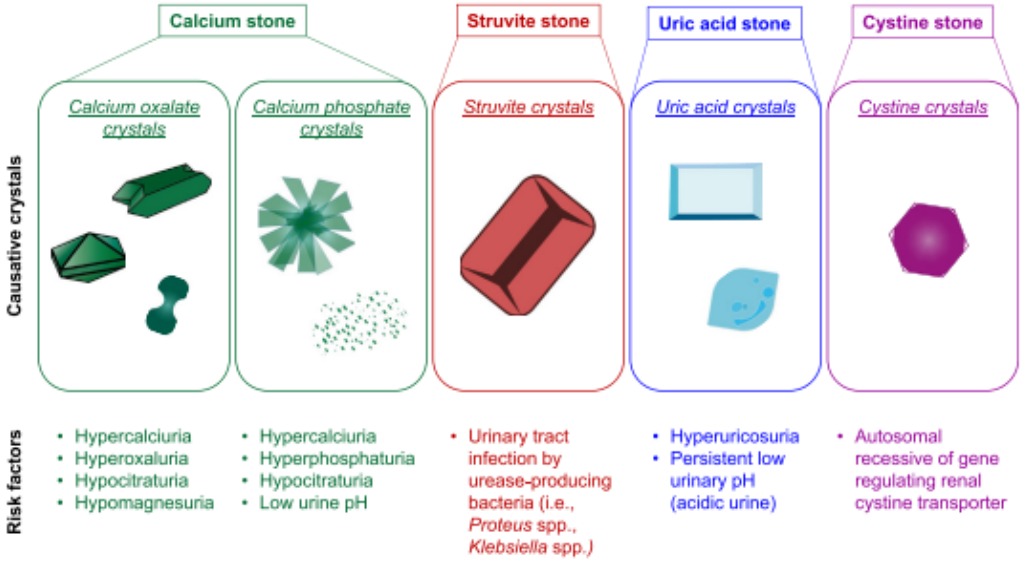

Figure 02: Stone categories and their risk factors **(Peerapen & Thongboonkerd, 2023)**.

I.3.2. Stone type

I.3.2.1. Calcium stones

As reported in various studies from different regions of the world, the most common mineral composition within all kidney stone types is calcium, making calcium-containing stones the most abundant stone, with a prevalence of 80% **(Coe, 2005; Alelign & Petros, 2018; Peerapen & Thongboonkerd, 2023; Tamborino et *al.*, 2024)**. Calcium stones are generally composed of either pure calcium oxalate (CaOX) (50%), pure calcium phosphate (CaP)(5%), or a combination of both with a percentage of 45% **(Tandon et *al.*, 2010; Tavasoli & Taheri, 2019; Tamborino et *al.*, 2024)**.

Based on its hydration status, CaOX has three crystalline forms: CaOx monohydrate (COM; $CaC_2O_4 \cdot H_2O$), CaOx dihydrate (COD; $CaC_2O4 \cdot 2H_2O$), and CaOx trihydrate ($CaC_2O4 \cdot 3H_2O$) **(Singh et *al*., 2015; Peerapen & Thongboonkerd, 2023)**. According to clinical data, COM is more frequently observed than COD and COT, as it is the most thermodynamically stable form of CaOX stones **(Alelign & Petros, 2018; Tavasoli & Taheri, 2019; Tamborino et *al*., 2024)**.

I.3.2.2. Struvite stone

Struvite or magnesium ammonium phosphate stones ($MgNH_4PO_4 \cdot 6H_2O$) also known as infection stones, occur with a prevalence of 10-15% among patients with chronic urinary tract infection (CUTI) due to the presence of urease-producing bacteria, such as *Proteus spp*, that cause urinary alkalinization, resulting in stone formation **(Alelign & Petros, 2018; Espinosa-Ortiz et *al*., 2019; Tamborino et *al*., 2024)**.

I.3.2.3. Uric acid stone

Uric acid stone or urate ($C_5H_4N_4O_3$) occurrence is approximately 3-10%. They are commonly present in the dihydrate form. It can be generated from endogenous as well as exogenous sources. Major risk factors for the formation of uric acid crystals include hyperuricosuria and persistently low urinary pH (pH < 5.05) …etc **(Frochot & Daudon, 2016; Peerapen & Thongboonkerd, 2023)**.

I.3.2.4. Cystine stone

With less than 2% of occurrence, these stones are considered a rare kidney stone type, cystine lithiasis ($C_6H_{12}N_2O_4S_2$) is caused by a genetic disorder affecting the transport of cystine. Under a urinary pH below 6.5 (normal level), cystine is relatively insoluble in urine, inducing its precipitation, crystallization and the formation of cystine stones from kidney stones **(Moussa et *al*., 2020; Peerapen & Thongboonkerd, 2023)**.

I.4. Molecular sources of nephrolithiasis

Based on available literature, several studies suggest that different molecular mechanisms could be involved in stone formation, such as, inflammation, oxidative stress, purine metabolism, and microbiome influence are significant part of the stone formation process **(Wigner et *al*., 2021; Jung et *al*., 2023; Tamborino et *al*., 2024)**.

I.4.1. Oxidative stress and inflammation

Reactive oxygen species (ROS) are highly reactive compounds. Under normal conditions (homeostasis), they play a crucial role in several normal physiological processes in the cell, notably in regulation signal cell transmission. However, an imbalance between the levels of these compounds (ROS) and physiological antioxidants can induce a state of oxidative stress (OS), which is involved in the appearance of different diseases, including kidney stones **(Kamata & Hirata, 1999; Dröge, 2002; Wigner et *al*., 2021)**.

According to evidence suggested by numerous studies, the presence of an interaction between preformed crystals may lead to the overproduction of ROS that triggers inflammation, which promotes the formation of more ROS **Figure 03**, causing a vicious cycle that leads to injury of the renal tubular epithelial cells (RTECs) and the formation of kidney stones. In the kidney, ROS are produced by two different pathways**(Khan, 2014; Khan et *al*., 2021; Tamborino et *al*., 2024).**

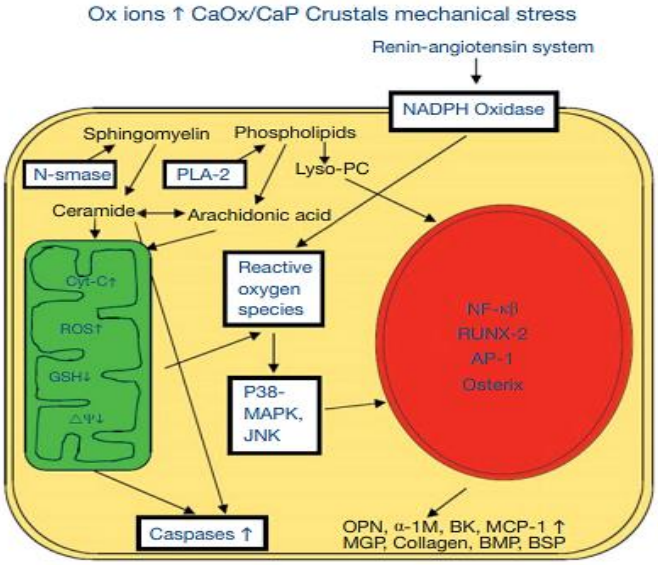

Figure 03: Oxidative stress and inflammation in the formation of nephrolithiasis (**Khan, 2014**).

The major one is the implication of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase. The NADPH oxidase is activated in the presence of CaOx crystals and Angiotensin II. This enzyme regroups six subunits of which the p47phox, p67phox, and p40phox, in the membrane activates the enzyme that will produce ROS. On the other hand, the deposition of CaOx crystals in the kidney causes damage to the mitochondria, known as the factory of ROS. The accumulation of ROS molecules will activate different cell death programs, leading to severe renal cell injury **(Khan, 2014; Khan et *al*., 2021; Tamborino et *al*., 2024).**

I.4.2. Purine metabolism

Purine metabolism is one of the metabolic pathways associated with the development of nephrolithiasis. Likewise, an enzyme deficiency linked to this metabolic process also contributes to the formation of stones **(Wigner et *al.*, 2021)**.As elucidated in **Figure 04** the purines adenine and guanine are converted to xanthine, which is then oxidized to produce uric acid. The levels of uric acid and xanthine depend critically on the enzymatic activities, indeed, deficiency of hypoxanthine-guanine phosphoribosyltransferase (HPRT) and xanthine oxidoreductase or dehydrogenase leads to excessive production of uric acid and xanthine respectively, which can lead to the formation of uric acid stones and xanthine kidney stones **(Williams,1990; Wigner et *al.*, 2021).** high levels of uric acid in the urine can lead to its deposition in kidneys, leading to nephrolithiasis.

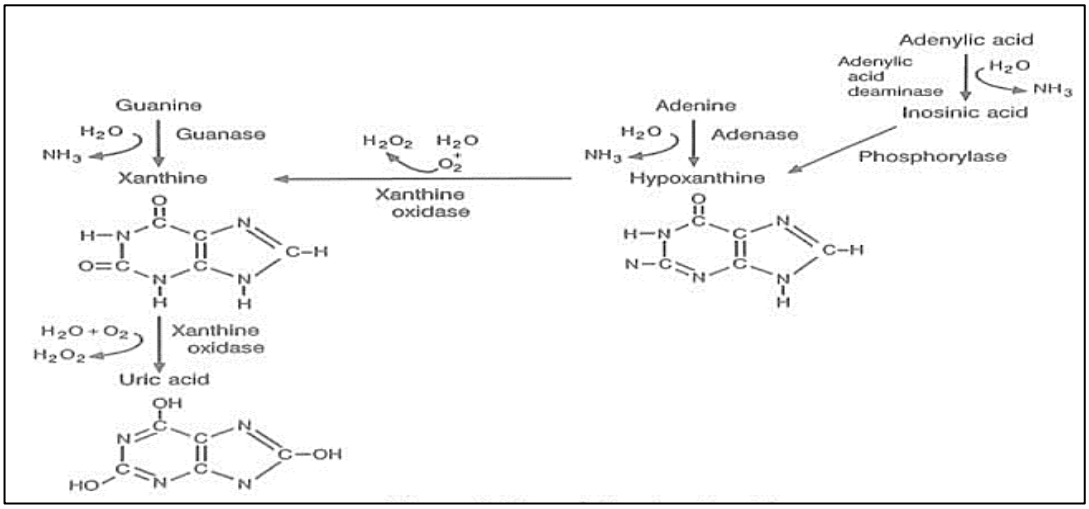

Figure 04: Purine metabolism **(Williams,1990)**

I.4.3. Microbiome nephrolithiasis

According to recent investigations the microbiome, tends to have a crucial role in the formation of some types of nephrolithiasis **(Jung *et al.*, 2023)**. While clinicians have associated the presence of bacteria in the urine as a sign of infection occurrence, it is not always the case *Lactobacilli*, *Bifidobacterium* and *Veillonellaceae* are found in the microbiome of healthy individuals, yet infection stones, such as magnesium ammonium phosphate, carbonate apatite, and ammonium urate, originate from the presence of specific microorganisms **(Jung *et al.*, 2023)**.

Recent studies have reported the association of enterobacteria, including *Escherichia coli*, in the formation of urolithiasis, as it affects calcium deposition in the urinary tract **(Barr-Beare et *al*., 2015; Jung et *al*., 2023)**. According to **Venkatesan et *al*. (2011)**, the presence of *E. coli* aggravates calcium oxalate deposition on the biofilm it produces, as these crystals bind to the bacteria and cause pyelonephritis.

Additionally, they produce citrate lyase, which decreases citrate levels and thus promotes calcium oxalatesupersaturation **(Barr-Beare et *al*., 2015; Jung et *al*., 2023)**. the microorganisms such as *Proteus mirabilis*, *Klebsiella pneumoniae* and *Serratia marcescens*, produce an enzyme called urease **(Jung et *al*., 2023)**.

Urease (urea amidohydrolase, EC 3.5.1.5), is a metalloenzyme requiring Nikel in its active site as a coenzyme. James B. Sumner, was the first to crystallize urease isolated from jack bean seeds (*Canavalia ensiformis*). This enzyme hydrolysis urea **figure 05 (Bichler et *al*., 2002; Espinosa-Ortiz et *al*., 2019)**. In the presence of water each urea is hydrolyzed into two ammonium and one carbon dioxide molecule, ammonium ions (NH^{4+}) and carbonate ions (CO_3^{2-}) can bind with different ions present in the urine thus forming microcrystals. Ammonium ions (NH^{4+}), when combined with magnesium (Mg^{2+}) and phosphate (PO_4^{3-}) ions, form the struvite stone. On the other hand, the binding of carbonate ions (CO_3^{2-}) with calcium (Ca^{2+}) and phosphate (PO_4^{3-}) ions produces carbapatite stone **(Bichler et *al*., 2002; Paliouras et *al*., 2012; Espinosa-Ortiz et *al*., 2019)**.

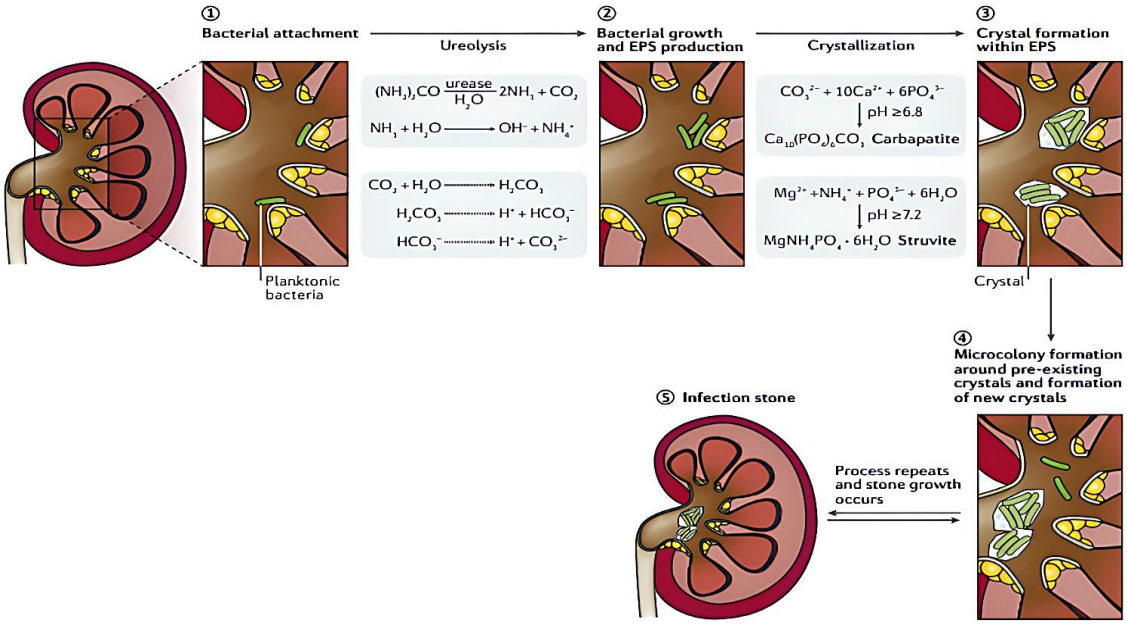

Figure 05: Urease mechanism in nephrolithiasis formation **(Espinosa-Ortiz et *al*., 2019)**.

I.5. Nephrolithiasis treatments

As mentioned before, nephrolithiasis is one of the most common urological diseases. Studies have shown that 35–50% of stone formers experience stone recurrence after their first acute kidney attack; currently, there is no definitive cure **(Devi et *al*., 2023; Tamborino et *al*., 2024)**.

However, several treatments and techniques can be implemented to offer relief to the patients by either breaking, detaching, dissolving, or removing the stone **(Devi et *al*., 2023; Tamborino et *al*., 2024)**. Common treatment strategies consist of:

I.5.1. Physical treatment methods

Surgical interventions, usually ureterolithotomy is used to remove the stone from the kidney **(Harmon et *al*., 1997)**. Other than surgery, extracorporeal shock wave lithotripsy uses different frequency waves to fragment the stone **(Torricelli et *al*., 2015; Talso, 2019)**. In the case of small-size stones flexible ureterorenoscopy is applied using external energy via a fine flexible fiber to break the stone **(Hyams et *al*., 2010)**.

I.5.2. Medication

Different types of stones require different medications as a treatment (Appendix I) summarizes some specific treatments for each type according to the Guidelines on Urolithiasis suggested by the EAU **(Tamborino et *al*., 2024)**.

I.5.3. Natural therapy

Conventional medications have several undesirable effects, and due to the high rate of recurrence, clinicians have been considering natural therapy as an alternative and maybe a complementary approach for the treatment of kidney stones. According to published data, high-potassium-containing fruit is highly recommended as it reduces the formation of stones. Several studies suggest the potential beneficial effect of different phytotherapeutic compounds like diuretic, litholytic, crystallization inhibition, anti-inflammatory, analgesic, anti-oxidant and antibacterial property against urolithiasis **(Cheraft-Bahloul et *al.*, 2017; Nirumand et *al*., 2018; Devi et *al*., 2023)**, but clinical trials are still needed to assess their effectiveness fully **(Moe, 2006; Devi et *al*., 2023)**.

I.6. Nephrolithiasis study models

Urinary lithiasis is considered a multifactorial disease **(Sakhaee, 2008; The Consensus Conference Group et *al*., 2016; Stamatelou & Goldfarb, 2023)**. The complexity and different interacting mechanisms rise a challenge for scientific research. Therefore, the implementation of different experimental study models is established to facilitate the comprehension and management of this disease by developing effective treatments **(Khan, 1997; Devi et *al*., 2023; Dong et *al*., 2024)**. A study model is a tool designed to mimic or create in a similar way the formation of stones and the mechanism behind this formation inside the human body to have a clearer vision of this disease **(Devi et *al*., 2023; Dong et *al*., 2024)**.

Several models belong to different categories to study nephrolithiasis **table I (Devi et *al*., 2023; Dong et *al*., 2024)**.

Table I: Nephrolithiasis study models **(Devi et *al*., 2023; Dong et *al*., 2024)**

Model	Description	References
In vitro	Used as a first step they generally, recreates one or more stages of the renal calculi formation process or studies the antioxidant and enzymatic pathways involved in the nephrolithiasis mechanism.	**Grases et *al*., 1998; Kanwal et *al*.,2019; Devi et *al*., 2023 ; Dong et *al*., 2024**
Semi–in vivo	Using part of living organisms to study the attachment and toxicity of kidney stones within the kidney. for example: RBC membrane, buccal cell membrane, human renal tubular epithelial (HK-2) cells, Madin-Darby Canine Kidney (MDCK) cells, normal rat kidney epithelial-like (NRK-52E) and spermatozoa	**Shckorbatov et *al*., 1995 ; Bigelow et *al*., 1996;)**, **Vollmer et *al*., 2019; Devi et *al*., 2023; Moretti et *al*., 2023 ; Dong et *al*., 2024**
In vivo	Using living organisms to understand the mechanism behind the formation and treatment of nephrolithiasis. However, some models outstand others. for example: the rat models induced by ethylene glycol (EG), gene knockout mouse and *Drosophila melanogaster*	**Singh & Hou, 2009; Devi et *al*., 2023 ; Dong et *al*., 2024**
In silico	Using bioinformatics and computational analytical tools such as molecular docking which implements different tools and algorithms to predict, at an atomic level, the interaction between a protein and a ligand and estimate the optimal position, orientation, and conformation of the ligand within the binding site of the protein. Thus, understanding the behavior and biochemical process behind this interaction.	**Meng et *al*., 2011; Agu et *al*.,2023**

However, it should be noted that there is no perfect study model, due to the complexity of the human body which poses a challenge in the research field **(Devi et *al*., 2023; Dong et *al*., 2024)**, hence, the crucial need to develop new models to analyze complex mechanisms and thus facilitate the understanding of the disease. Furthermore, the objective of our research is to be able to applicate some models reported in literature and determine a new model that will allow us to study the cellular toxicity of calcium oxalate monohydrate (COM) by using extracts of pomegranate plant.

I.7. *Punica granatum*:

I.7.1. Description

Punica granatum L., commonly known as pomegranate, is a Latin word where "*pome*" stands for apple and "*granate"* meaning-many-seeded. It is a plant originating from the Mediterranean basin **(Guerrero-Solano et *al*., 2020 ; Maphetu et *al*., 2022)**. In Algeria, according to locals, it is referred to as Tha'rmant or Rouman, as it is in the Arabic language. The pomegranate plant is a small bushy tree that only grows 4-5 m long. The trunk of the tree is covered by brown-reddish bark that transforms to grey as the plant grows older; it is also characterized by bright red-colored flowers blooming in the summer and eventually turning into a fully grown pomegranate fruit **(Holland et *al*., 2009)**. The fruit peel can be green, pink, reddish, or dark red in color. Data indicates that the peel's thickness varies from tree to tree and is typically between 1.5 and 4.24 mm **Figure 09.** A thin membrane divides the fruit's interior into sections, each of which contains several tiny seeds encased in a juicy pulp sac that forms the actual edible portion of the pomegranate. The fruit is usually harvested in the period between September and November **(Erkan & Kader, 2011)**.

Figure 09: *Punica granatum* L. fruit (A), *Punica granatum* flower (B) **(Original)**

I.7.2. Taxonomy

According to botanical studies *Punica granatum* is classified as shown in **Table II** below.

Table II: Taxonomic positioning of *Punica granatum* L. **(Panth et *al.*, 2017 ; Kumari et *al.*, 2021)**.

Classification	Denomination
Kingdom	Plantae
Division	Tracheophyta
Class	Magnoliopsida
Order	Myrtales
Family	Lythraceae
Genus	*Punica*
Species	*Punica granatum*
Common Name	Pomegranate

I.7.3. Uses in traditional medicine

Since prehistoric times (4000–3000 BCE), the benefits of pomegranate fruit have been acknowledged. In Ayurvedic medicine, all parts of the pomegranate are utilized including roots, bark, flowers, fruits, and leaves. The bark was historically used to treat diarrhea, dysentery and ulcers as they believed it had powerful anthelminthic, vermifugic, and anti-parasitic properties **(Eddebbagh et *al.*, 2016)**. In Indian culture, an infusion of pomegranate peel, bark, and flowers was used to treat intestinal worms, diarrhea, nose hemorrhage, and ulcers **(Karimi et *al.*, 2017).** Furthermore, according to **Darabinia et al. (2016**), Abu-Ali Sina claimed that "pomegranate bark is useful for treating inflammation, liver, cough, and soreness, pomegranate flower cuts the bleeding and strengthens the gums, and pomegranate powder treats the old wounds."

I.7.4. Phytochemicals in pomegranate

According to the data *Punica granatum* is a rich source of various polyphenols, including ellagitannins, gallotannins, ellagic acids, gallagic acids, catechins, anthocyanins, ferulic acids, and quercetins. It also has a great content of organic acid, mainly citric acid (**Erkan & Dogan, 2018; Zeghad et *al.*, 2022).** The studies on the composition of pomegranate peel confirmed the presence of over 48 compounds, such as alkaloids, anthocyanins, anthocyanidins, tannins, flavonoids, phenolics, proanthocyanidins, sterols, terpenes, and xanthonoids, while the flowers also contain tannins such as ellagic acids, punicatannin C, and garlic acid. Additionally, it contains terpenoids and flavonoids.

Material and Methods

II. Material and methods

II.1. Material

II.1.1. Plant material *Punica granatum* L.

II.1.1.1 The peel and flowers of *Punica granatum* L. extracts

The fruit of the pomegranate was harvested in late October 2017 in Sidi Ayad (Sidi Aich), Wilaya of Bejaia, while the flowers were collected during the month of May, 2019 at Ain Touta, Wilaya of Batna. An identification of the plant was done according to the ethnobotanical literature. The drying process involved placing the peel of *Punica granatum* L. at room temperature in a dry airy location away from light. At the same time, the flowers were placed outdoors to air dry. Following this, both parts of the plant were finely ground using an electric grinder.

For *Punica granatum* peel, two ethanolic extractions were carried out according to the method of Cheraft-Bahloul et *al*. (2017). The first one is subjected to a delipidation step, where the powder was macerated in n-hexane (1/10, m/v) for 24 hours. The powder recovered from this step was then introduced to ethanol (1/10), and successive exhaustion was performed under continuous shaking at room temperature for 24 hours. After decantation, the extracts were recovered, and the ethanol was then evaporated using a steam rotor. On the other hand, the second extraction was prepared by introducing directly the plant's powders to ethanol and followed the same steps as before for the extraction without delipidation.

For flowers part, an infusion method according to Jiménez-Zamora et *al*. (2016), was used with minor modifications. This involved adding 5g of *Punica granatum* flower's powder to 100 ml of boiling water. The mixture was left at room temperature for 4 hours to allow for the infusion process. The mixture was first filtered using Whatman filter paper No.1, then centrifuged at 5000 rpm for 10 min. The supernatant was then filtered onto filter paper and the filter was recovered in a sterilized vial stored at a low temperature and protected from light. Finally, the extracts of fruit peel (PGD and PGND) and flowers (PGF) of *Punica granatum* L. were weighed and stored at -20 °C until their use in the different tests.

II.1.2. Biological material

For this study, animal tissue of mature goats (*Capra hircus*) procured from the slaughterhouses in Bejaia city were used. Testis was collected from a local butcher shop, kept at 4 °C and then transported with an icebox to the laboratory.

II.2. Methods

II.2.1. Ferric Reducing Antioxidant Power (FRAP) assay of *Punica granatum* extracts

The Ferric Reducing Antioxidant Power (FRAP) of *Punica granatum* extract was conducted using the Trolox equivalent antioxidant capacity (TEAC) based on the work of Firuzi et *al*. (2005). This method is based on the reduction of the ferric complex (Fe3+-TPTZ) to its colored ferrous form (Fe2+-TPTZ) via the presence of an antioxidant as described in **Figure 07**.

Briefly, the FRAP solution was prepared by combining 10 ml of acetate buffer 300 mM, at pH 3.6 adjusted via the addition of acetic acid, then mixed with 1 mL of ferric chloride hexahydrate 20 mM, dissolved in distilled water and 1 mL of 2,4,6-Tris(2-pyridyl)-s-triazine (TPTZ) (10 mM) dissolved in HCl (40 mM). This solution was stored in a dark location. The analyses were carried out in a 96-well microplate in triplicate, and the blank contained 200 µL of the FRAP solution. Whereas, each test well contained 180 µL of the FRAP solution along with 20 µL of the different extracts making a total volume of 200 µL. After 10 minutes incubation at 37°C, the absorbance was read at 595 nm with a temperature control set at 37°C.

The results were carried out in equivalence of mg of Trolox per gram of extract (mg eqTrolox/ g extract), according to a calibration curve made in the same conditions, using TROLOX as a reference molecule (Annex II).

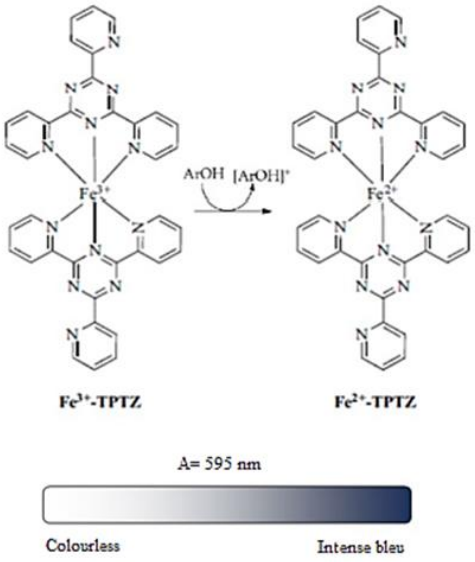

Figure 07: Principal of the FRAP method **(Munteanu & Apetrei, 2021)**

II.2.2. *In vitro* Enzymatic tests

II.2.2.1. Xanthine oxidase inhibitory activity of *Punica granatum* extracts

Bovine milk xanthine oxidase was used to assess the inhibitory effect of *Punica granatum* extract using the method described by **Owen & Johns (1999)** with some modifications. This method is based on the formation of uric acid from the interaction between xanthine oxidase and xanthine. The assay mixture was prepared by adding 200 µL of xanthine solution to 1760 µL of phosphate buffer (pH 7.5), followed by the addition of 20 µL of *Punica granatum* extracts at different concentrations. For PGF concentrations from 12.5, 25, 50, 100 to 150 µg/mL were implemented, as for the PGPnd extract we used concentrations of 25, 50, 100, 150 and 200 µg/mL, while for the PGPd concentrations ranged from 12.5, 25, 50, 100 to 500 µg/mL. For each extract and concentration, a blank was prepared by substituting the extract with methanol. The reaction was initiated by adding 20 µL of enzyme solution (0.2U/mL). The kinetics of uric acid production were monitored spectrophotometrically at 295 nm for 3 minutes. The assays were carried out in triplicate against allopurinol, which was used as a reference molecule. The inhibition percentage was calculated using the following equation:

$$\textbf{Inhibition}\ (\%) = \frac{(\boldsymbol{Absorption\ of\ positive\ control}) - (\boldsymbol{absorbance\ of\ the\ extract})}{(\boldsymbol{Absorption\ of\ positive\ control})} \times \mathbf{100}$$

II.2.2.2. Urease inhibitory activity of *Punica granatum* extracts

Weatherburn protocol (1967) was adopted to assess the inhibitory effect of *Punica granatum* extracts on urease. This test is based on monitoring the formation of ammonium from the reaction of urease with urea. To perform this test, 25 µL of urease enzyme (4U) pre-prepared in a phosphate buffer solution was injected into a 96-well microplate. 15 µL of *Punica granatum* extracts at concentrations of 11.7, 23.4, 46.875, 93.75, 187.5 and 375 µg/mL along with the standards (boric acid) are then added to the enzyme. The plate is then incubated at 30°C for 15 minutes. After the incubation, 40 µL of urea (100 mM) are added and re-incubated at 36°C for 30 minutes. Subsequently, 50 µL of phenol (1% phenol + 0.005% sodium nitroprusside) are added.

The absorption is measured at 630 nm for 50 minutes at 36 °C. Each tested concentration was performed in triplicate, and their inhibition percentage was calculated using the following equation:

$$\textbf{Inhibition}\ (\%) = \frac{(\boldsymbol{Absorption\ of\ positive\ control}) - (\boldsymbol{absorbance\ of\ the\ extract})}{(\boldsymbol{Absorption\ of\ positive\ control})} \times \mathbf{100}$$

II.2.3. *In silico* enzymatic tests

II.2.3.1. Molecular docking studies

Molecular docking was implemented to estimate the interaction of *Punica garantum* extract as a potential inhibitor of both urease and xanthine oxidase in the AutoDock (v4.2) program. The structure of both enzymes was downloaded from the Protein Data Bank (http://www.rcsb.org/pdb), XO (PDB ID: 3NVW) and Jack bean urease (PDB ID: 4H9M), while the 3D structure of *Punica garantum* specific compounds namely punicalagin and ellagic acid were taken from the PubChem data base. Allopurinol and boric acid were used as reference molecules for xanthine oxidase and urease respectively. The AutoDock Tool (ADT), included with the MGLTools package (version 1.5.6) was used to add charges and polar hydrogen atoms and set up rotatable bonds to prepare and optimize the protein and ligands **(Morris et *al*.,2009)**.

AutoDock Tool was used to create PDBQT files for the protein and ligands at that same moment. AutoDock4 was used to execute the molecular docking. The protein's active binding site, which was obtained by eliminating the ligand, was selected as the grid center. To include all atoms in the ligand set, the center of grid box dimensions was chosen. The grid box site in XO (3NVW) was set at (x= 88.201, y= 8.976, z= 19.104), using a grid of 80 Å, 80 Å and 80 Å and a grid spacing of 0.5Å. For urease (4H9M) grid box was set at (x= 19.067, y= -56.327, z= -21.334), using a grid of 70 Å, 66 Å and 64 Å and a grid spacing of 0.375 Å. The protein macromolecules were kept stiff throughout the docking simulation, and the docking parameters were determined using the Lamarckian Genetic Algorithm 4.2 . The number of executions of the genetic algorithm was set to 50, and the other bonding parameters were preserved by default. The optimal protein-ligand conformation was identified using the AutoDock4.2 scoring tool based on the highest binding affinity.

BIOVIA Discovery Studio Visualizer 4.1 was used for post-docking analysis and to align and override complexes anchored on the reference co-crystallized protein complex to calculate comparative mean square gap values (RMSD).

II.2.4. Evaluate anti urolithiasic activity of *Punica granatum* extracts

II.2.4.1. Measurement of turbidity

The measurement of turbidity is carried out by a spectrophotometric method, according to Cheraft-Bahloul et *al*. (2017). The principle of turbidimetry is measuring the optical density of a cloudy state that exists in a solution. This test was performed to evaluate the effect of *Punica granatum* extracts on aqueous COM suspension. A 96-well plate was used, and the test was carried out in triplicate. Each well contained a ratio of 1:1 (v/v) of COM solution along with the *Punica granatum* extracts at concentrations of 6.25, 12.5, 25, 50, 100 and 200 µg/mL, then continuous agitation was performed. The optical density was measured using the microplate reader (Synergy HTX multi-mode Reader Biotek) at 660 nm at different times.

II.2.4.2. Semi-*in vivo* models test

- **Epididymal semen collection**

Sperm was collected using the retrograde flushing method according to **Martinez-Pastor et *al*. (2006)**. This method consists of separating the epididymis of the testis and then cleaning it. After isolating both cauda and vas deferens from the epididymis, we pursued by eliminating all blood vessels from the surface of the cauda epididymis. The cauda was then rinsed and wiped. Then, using a syringe loaded with 1ml of extender we generated pressure through a perfusion from the vas deferens and cauda. The sperm flushed out from a cut performed in the distal cauda, and air was injected afterwards to insure the recuperation of all the contents in the cauda epididymis. The samples were collected in a 1.5 ml Eppendorf.

- **Motility assay**

The effects on spermatozoa motility parameters were evaluated using the CASA system (Sperm Class Analyzer, S.C.A. v 3.2.0, Microptic S.L., Barcelona, Spain). The toxicity of COM was assessed using the spermatozoa study model. Different samples at different concentrations were prepared. After the determination of the toxic concentration, a protection assay using *Punica granatum* aqueous extract was initiated. a volume of 10 µL of the sperm dilution was added to a mixture of 45µL of COM solution at a concentration of 500µg/ml along with *Punica* extracts with a concentration range from 50 to 1000 µg/mL. For the motility assay10 µL was loaded in an analysis chamber (Makler Counting chamber, Sefi-Medical Instruments ltd., Biosigma S.r.l., Italy). Using a phase contrast microscope at a 10X field, we analyzed spermatozoa kinematics. The parameters measured were straight-line velocity (VSL), Curvilinear velocity (VCL), and Average path velocity (VAP).

II.2.5. Statistical analysis

The results of the antioxidant activity as well as the turbidity and the *in vitro* enzymatic inhibitory assays were expressed as a mean of triplicates ± standard deviation (SD), and analyzed using Prism 8 software (GraphPad Software, Inc., San Diego, CA, USA) with the ordinary one-way ANOVA test.

Whereas the results of the semi *in vivo* models were expressed by a mean of triplicates ± standard error of the mean (SEM) by application of the F test of variance equality, data were analyzed using Statview 4.02 software (Abacus Concepts Inc., Berkeley, CA, USA). The difference is considered to be significant for $p< 0.05$ (*), $p < 0.01$ (**), $p< 0.001$(***) and p< 0,0001(****).

Results and discussion

III. Results and discussion

III.1. Ferric Reducing Antioxidant Power (FRAP) assay of *Punica granatum* extracts

Using FRAP assay, reducing power was measured in the peel and flowers of *Punica granatum* extracts. In this assay, the antioxidant compounds act as reducers in colorimetric reaction, forming the ferrous complex (Fe(II)-TPTZ) by reducing the ferric tripyridyl-triazine complex (Fe(III)-TPTZ). This complex is characterized by a blue color that can be measured using spectrophotometry at a wavelength of 595 nm **(Pulido et *al*., 2000; Li et *al*., 2006; Benchagra et *al*., 2021)**. The results were expressed in equivalent trolox (mg Eq Trolox/mg extract) using a Trolox standard curve.

As shown in **Figure 08**, both the aqueous flower extract (PGF) and the ethanolic non-delipidated peel extract (PGP nd) exhibit high reducing activity (160.19±3.035 mg Eq Trolox /mg of extract and 153.80±7.847 mg Eq Trolox /mg of extract, respectively) (*P> 0.05*). However, the ethanolic delipidated peel extract (PGPd) presented a low reducing power of 78.25±0.234 mg Eq Trolox /mg of extract (*P*<0.0001).

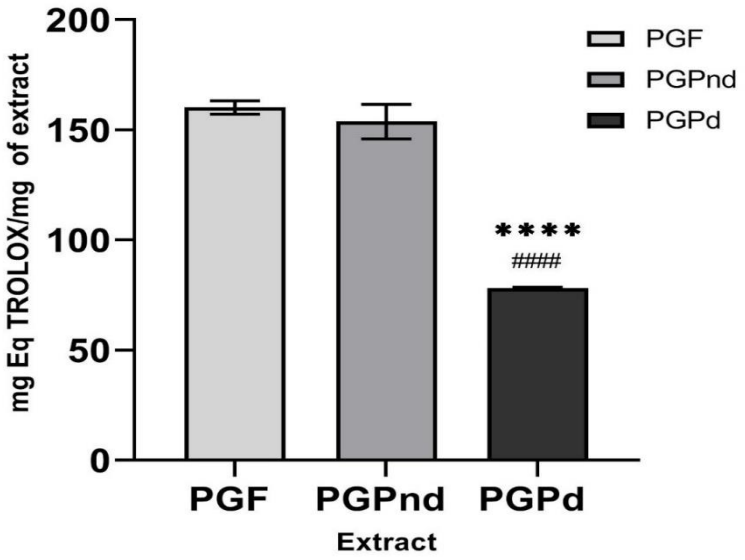

Figure 08: Ferric Reducing Antioxidant Power (FRAP) assay of peel and flower of *Punica granatum* extract*s*. PGF: aqueous flower extract, PGPnd: ethanolic non-delipidated peel extract, PGPd: ethanolic delipidated peel extract. Values are expressed as mg equivalent TROLOX/mg extract expressed as mean ± SD (n = 3). Significance (∗p < 0.05; ∗∗p < 0.01; ∗∗∗p < 0.001 ∗∗∗∗p < 0.0001) compared to the PGF extract and significance (#p < 0.05; ##p < 0.01; ###p < 0.001 ####p < 0.0001) compared to the PGPnd extract by one way ANOVA test.

The studies conducted by **Rummun et *al.* (2013)** and **Fellah et *al.* (2018)** indicated that the flower extract exhibited the greatest ability to reduce ferric ions, followed by the peel extract. In addition, **Peršurić et *al.* (2020)** found that the ethanolic peel extracts demonstrated significant antioxidant activity, with values ranging from 100.25 to 176.60 µmol Eq Trolox /100 g of extract.

Our findings were consistent with a study conducted by **Hajimahmoodi et *al.* (2013)** on flower of *P.granatum* extracts, which concluded that it had the highest ferric-reducing capacity. **Petrova et *al.* (2021)** found that the aqueous flower of *P.granatum* extracts had a significant reducing power of 655.6 ± 4.7 mM TE/g extract.

Regarding the dilapidated extract, it showed a rather low ferric reduction power of 78.25±0.234 mg Trolox Eq/ mg of extract. A study conducted by **Karthikeyan & Vidya (2019)** showed that the use of hexane as a solvent for peel extraction resulted in the lowest antioxidant power, whereas the ethanolic extract exhibited the best antioxidant power.

The behavior of the aqueous flower extract and the ethanolic non-delipidated peel extract was similar, in comparison with the delipidated peel extract. According to the literature **(Rummun et *al.*, 2013; Gigliobianco et *al.*, 2022; Sweidan et *al.*, 2023)**, the difference in behavior is due to the phenolic content. However, findings indicate that the efficiency of an extract is not only based on the amount of phenolic compounds; synergistic activities between the compounds are highly valuable for antioxidant activities **(Rummun et *al.*, 2013)**.

In our case, the delipidation process significantly lowered the antioxidant power of the ethanolic extract, although according to data, the ethanolic peel extract presents high antioxidant properties. Since ethanol allows the extraction of all phytochemical classes found in the peel (phenols, flavonoids, anthocyanins, coumarins, quinones, tannins, saponins, steroids, triterpenoids, and alkaloids), this correlation between a high amount of phytochemical agents and the antioxidant activities is verified **(Gil-Martín et *al.*, 2022; Sweidan et *al.*, 2023)**.

The studies on all parts of the pomegranate classified the flower as the most abundant in bioactive compounds. The same order was obtained for the FRAP assay: flower > peel > leaf > stem > seed **(Ardekani et *al*., 2011; Rummun et *al*., 2013)**. Our results confirm and verify these conclusions, despite slight concentration varieties that could be due to the geographical region. The behavior of the extracts is in concordance with other studies.

III.2. *In vitro* enzymatic assay

III.2.1. Xanthine Oxidase Inhibitory Activity of *Punica granatum* extracts

Xanthine oxidase (XO, E.C.1.1.3.22) is an enzyme involved in purine metabolism, producing uric acid as an end product. This reaction can be performed *in vitro* with the addition of xanthine and an inhibitor to measure the inhibition percentage, which can be monitored by observing the kinetics of uric acid formation using spectrophotometry at a wavelength of 295 nm **(Owen & Johns, 1999)**

Different concentrations of PGF (12.5 to 150 µg/mL), PGPnd (25 to 200 µg/mL) and PGPd (12.5 to 500 µg/ mL) extracts were tested for their inhibitory effects on xanthine oxidase. Allopurinol was used as a reference molecule (0.1 and 10 µg/mL).

According to **figure 09**, all *P.granatum* extracts showed a dose-response inhibition activity on xanthine oxidase. The PGF extract had the highest inhibition activity at a concentration of 150 µg/mL, followed by PGPnd at 200 µg/mL, and PGPd at 500 µg/mL Allopurinol, on the other hand, achieved 100% inhibition at a concentration of 10 µg/mL. This variation in inhibition activity profiles based on concentration demonstrates a dose-response relationship.

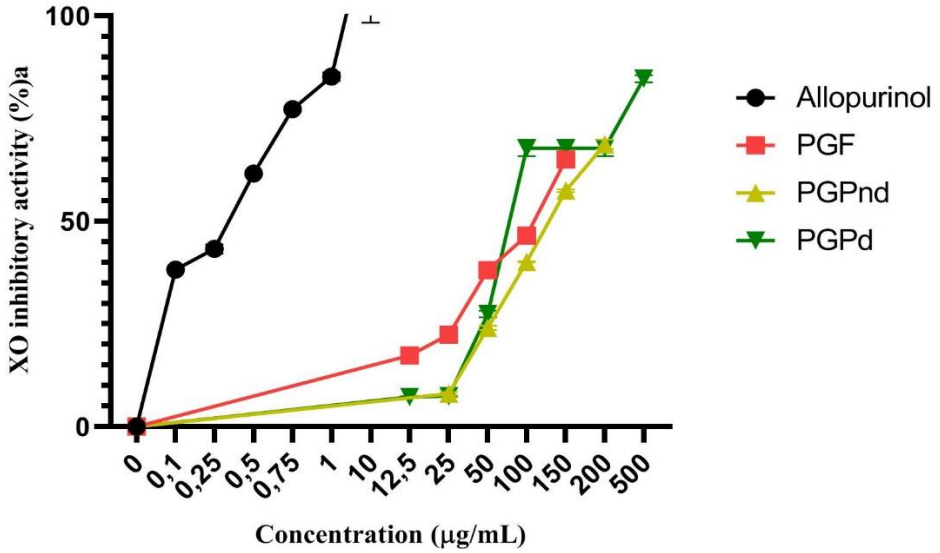

Figure 09: Xanthine Oxydase inhibitory activity of *P. granatum* extracts and allopurinol at various concentrations. PGF: aqueous flower extract, PGPnd: ethanolic non-delipidated peel extract, PGPd: ethanolic delipidated peel extract. Value are expressed as the mean ± SD (n = 3). Significance (∗p < 0.05; ∗∗p < 0.01; ∗∗∗p < 0.001 ∗∗∗∗p < 0.0001) compared to allopurinol is expressed by one way ANOVA test.

Wong et *al*. (2014) found that the methanolic extract from the peel of *P. granatum* showed very little or no ability to inhibit xanthine oxidase at a concentration of 100 µg/mL. Our results surpassed theirs, as both of the ethanolic extracts the non-delipidated and the delipidated peel extracts, revealed an inhibition rate of 40.12 ± 0.091-and 67.80 ± 1.97%, respectively However, the aqueous flower extract showed an inhibition of 65.03 ± 0.80% at only 150 µg/mL.

In a recent work conducted by **Li et *al*. (2024)**, an ethanolic flower extract was used, resulting in an inhibition rate of 76.22 ± 2.59% at a concentration of 200 µg/mL. This behavior suggests that flower's extract has higher inhibitory effects against xanthine oxidase than previous studies.

Based on inhibition percentage at various concentrations, the half maximum inhibitory concentration (IC_{50}) of *P. granatum* extracts and allopurinol values were determined. IC_{50} indicates the concentration required for an extract to inhibit 50% of the enzyme activity. The results are presented in **Table III**. The IC_{50} values are classified as the most active as follow: Allopurinol> PGF> PGPd > PGPnd. When compared to the IC_{50} value of allopurinol (2.25±0.015 µg/mL), a reference substance, all the extracts showed a significant differences, but their values remain low and reflect

a powerful inhibitory effect (38.11±7.835 , 62.97±3.915 and 71.50±8.22 µg/mL for PGF, PGPd and PGPnd, respectively)

Table III: IC50 values of xanthine oxidase inhibitory activity of *P. granatum* extracts and allopurinol

Extract	IC_{50}(µg/ mL)	R^2
PGPnd	71.50±8.22 ****	0.9617
PGPd	62.97±3.915 ****	0.9899
PGF	38.11±7.835 ***	0.9041
Allopurinol	2.25±0.015	0.9384

PGF: aqueous flower extract, PGPnd: ethanolic non-delipidated peel extract, PGPd: ethanolic delipidated peel extract. Value are expressed as the mean ± SD (n = 3). Significance (*p < 0.05; **p < 0.01; ***p < 0.001 ****p < 0.0001) compared to allopurinol expressed by one way ANOVA test.

Allopurinol [4-hydroxypyrazolo (3,4-d) pyrimidine] is a standards xanthine oxidase inhibitor, approved for treating hyperuricemia conditions since 1966 **(Wang et *al*., 2014; Vijeesh et *al*., 2021; Tran et *al*., 2024)**. It is hydrolyzed by xanthine oxidase (XO) into oxypurinol, which then binds to the reduced state of the molybdenum (IV) site in the enzyme and thus inhibits competitively the uric acid formation **(Chen et *al*., 2016)**. However, this strong inhibitor causes several side effects **(Okamoto et *al*., 2008; Chen et *al*., 2016)**. Due to these side effects **(Mcinnes et *al*., 1981)**, there is increasing interest in using plants as an alternative. *P. granatum*, known for its richness in bioactive compounds, is used to treat several diseases.

Previous studies have shown that *P. granatum* extract exhibits inhibitory effect against xanthine oxidase. The study conducted by **Li et *al*. (2024)** revealed that the aqueous extract of *P granatum* flowers had inhibitory effect against xanthine oxidase. These findings concords with our results.

A study conducted by **Wang et *al*. (2014)** found that the methanolic extract of *P.-granatum* did not exhibit any action. Nevertheless, our findings demonstrated that both delipidated and non-delipidated ethanolic peel extracts exhibited inhibitory efficacy. This discrepancy may be attributed to differences in the method of extraction.

P. granatum is known to be rich in secondary metabolites, particularly phenolic compounds. Researches have demonstrated that these compounds, including flavonoids, have inhibitory activity against xanthine oxidase.

Atmani et *al.* (2009) reported that flavonoids containing a hydroxyl group at position C-5 and C-7, and a planar structure with a double bond between C-2 and C-3, exhibited XO inhibitory activity. This was further confirmed by **Wang et *al.* (2014)**.

On the other hand, **Nagao et *al.* (1999)** mentioned that substitution of the hydroxyl group at C-3 and C-7 with glycoside or a methyl group reduces the inhibitory activity against xanthine oxidase. They also stated that quercetin and kempeferol, potent xanthine oxidase inhibitors found in *P. granatum*, have lower inhibition activity in their glycoside state. This suggests that glycosylation of certain positions within the flavonoid structure might lead to interference with the enzyme binding process, resulting in lower inhibitory activity **(Nagao et *al.*, 1999)**.

Moreover, **Liu et *al.* (2020)**, reported that different flavonoids are affected in different ways by the changing process. The formed products dictate their xanthine oxidase inhibition activity. For example, the glycosylation of quercetin at position C-3 into isoquercitrin resulted in high inhibitory activity **(Liu et *al.*, 2017)**, while the glycosylation of the same molecule at positions C-3 and C-4' into quercetin-3,4′-O-diglucoside actually lowered the inhibitory activity **(Nile et *al.*, 2017)**.

Similarly, the glycosylation of Kaempferol at positions C-3 and C-7 into kaempferitrin lowered the inhibitory activity, as did the methylation of the same compound at position C-4' into kaempferide **(Yuan et *al.*, 2019)**. Luteolin, another compound found in *P. granatum*, exhibited inhibitory activity when glycosylated at C-4' into luteolin-4′-O-glucoside **(Zhang et *al.*, 2016)**, while glycosylation at C-6 and C-8 into luteolin-6-C-glucoside significantly lowered the inhibitory activity **(Materska, 2015).**

III.2.2. Urease Inhibitory Activity of *Punica granatum* Extracts

The urease enzyme (urea amidohydrolase; EC 3.5.1.5) catalyzes the hydrolysis of urea to ammonia and carbon dioxide. This reaction can be replicated, *in vitro* to study the inhibitory activity of *P.granatum* extracts. The inhibition process can be monitored using spectrophotometry by observing the kinetics of ammonia formation at a wavelength of 630nm **(Weatherburn, 1967)**.

In this study, *P.granatum* extracts (PGF, PGPnd, PGPd) were used at concentrations ranging from 11.7 to 375 µg/mL. Boric acid was used as a reference compound at 75 to 1050 µg/mL. According to the results **figure 10**, all the extracts showed a similar variation profile with a dose-response urease inhibition activity.

Both PGF and PGPnd extracts exhibited maximum inhibition at a concentration of 93.75 µg/mL, while PGPd extract showed maximum inhibition at 187.5 µg/mL, achieving 100%. In comparison, boric acid required a concentration of 1050 µg/mL to achieve an inhibition of 79.21±4%.

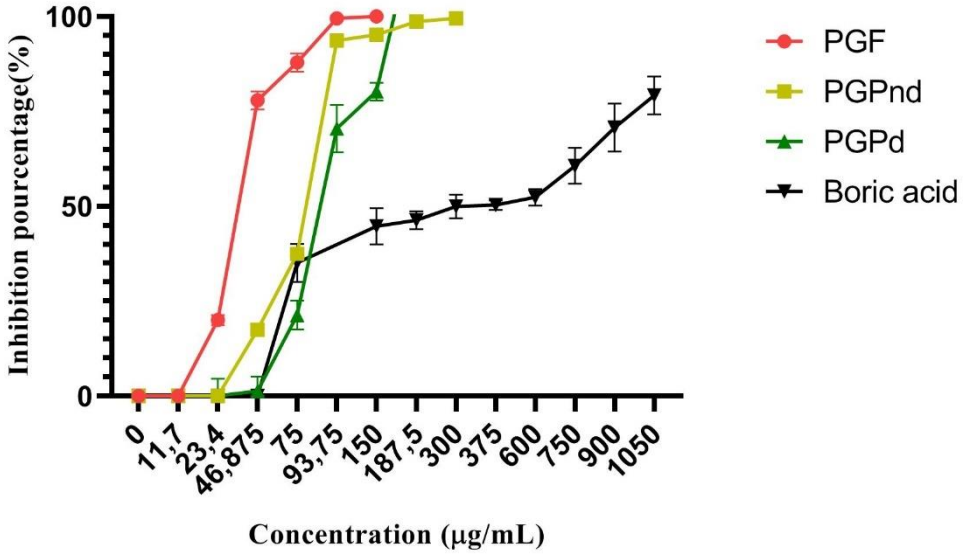

Figure 10: Urease inhibitory activity of *P.granatum* extracts and boric acid at various concentrations. PGF: aqueous flower extract, PGPnd: ethanolic non-delipidated peel extract, PGPd: ethanolic delipidated peel extract. Value are expressed as the mean ± SD (n = 3). Significance ($^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$ $^{****}p < 0.0001$) compared to boric acid is expressed by one way ANOVA test.

In order to determine the IC_{50} value, non linear regressions were analyzed between the tested extracts based on the exposed inhibition percentage in conjunction with boric acid. The results are presented in **Table IV**. According to statistical analyses the difference from both the PGP delipidated extract and the PGP non-delipidated extract was non-significant. The PGP non-delipidated extract had the best IC_{50} value of 32.61±0.625 µg/mL compared to the delipidated extract (P< 0,01). However, when compared to boric acid, a standard substance, all extracts including PGF the PGP delipidated and non-delipidated extracts had a very highly significant difference (P < 0.0001), as the boric acid had the highest IC*50* value of 499.5±29.3 µg/mL.

Table IV: IC50 values of *P.granatum* tested extracts and boric acid

Extract	IC_{50} (µg/mL)	R^2
PGPnd	32.61±0.625****	0.9910
PGPd	79.56±4.59****	0.9986
PGF	59 ±1.155****	0.9989
Boric acid	499.5±29.3	0.80

PGF: aqueous flower extract, PGPnd: ethanolic non-delipidated peel extract, PGPd: ethanolic delipidated peel extract. Value are expressed as the mean ± SD (n = 3). Significance ($^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$ $^{****}p < 0.0001$) compared to boric acid expressed by one way ANOVA test.

In **2012, Nabati and colleagues** studied the inhibitory activity of several different plants including *Punica granatum*. According to their findings, the methanolic extract of pomegranate peel demonstrated an inhibition rate of 99.90±0.01% at a concentration of 1000 µg/mL. However, the results of our ethanolic extracts, both delipidated and non-delipidated, surpassed theirs. PGP delipidated extract tested in the current study exhibited an inhibition rate of 100.16±0.96% at 187.5 µg/mL, while the PGP non-delipidated extract showed even better results with an inhibitory rate of 93.69±0.81%, at only a concentration of 93.75 µg/mL

Additionally, they also investigated the inhibitory effect of methanolic flower extract of pomegranate, obtaining an inhibition activity of 99.90±0.01% at a concentration of 1000 µg/mL **(Nabati et *al*., 2012)**. In contrast, our aqueous flower extract demonstrated an inhibitory rate of 99.48 ± 0.39% at a concentration of 93.75 µg/ml. These results indicate that our extract is more potent than the methanolic extract, likely due to the different extraction methods and the origin of the plant.

In another study, **Bai et *al*. (2015)** used methanolic and aqueous extracts of *Lawsonia inermis* L., a plant from the same family as *P. granatum*. Their results showed low inhibition activity of urease for both extracts at a concentration of 1000 µg/mL, with 5.83 ± 0.02 and 4.32 ± 0.01% for methanolic and aqueous extracts, respectively. Our results clearly surpassed theirs, which could be due to the richness of *P. granatum* on bioactive compounds, in comparison to *Lawsonia inermis*.

In terms of IC_{50}, our ethanolic peel extract gave great results with the PGP delipidated and PGP non-delipidated showing an IC_{50} of 32.61±0.625 and 79.56±4.59 µg/mL, respectively. Meanwhile, the methanolic peel extract showed an IC_{50} of 1484 ± 0.10 µg/mL. Similarly, flower's extract performed better with an IC_{50} of 59±1.15 µg/mL compared to an IC_{50} of 1331±0.11 µg/mL for their methanolic extract.

The results demonstrate the effectiveness of the studied *P. granatum* extracts when compared to other species, origins, and even to the reference compound, boric acid. In fact, according to **Krajewska & Brindell (2016),** boric acid acts as a standard competitive inhibitor of urease. The B(OH)3 form, binds to Ni ions with two O-atoms in the active site of the enzyme, while the third one heads toward the opening of the active site. It is important to note that boric acid isn't safe for continuous use as it can cause adverse side effects with excessive exposure, as confirmed by a recent study by **Ismail (2022)**. Therefore, finding an alternative is highly recommended.

P. granatum is a plant rich in secondary metabolites and has proven to be efficient in inhibiting urease, according to research conducted by **Biglar et *al.* (2021)**. Indeed, the results obtained in this study align with their findings. This activity is suggested to be related to the phytochemical composition of *P. granatum*, as it is rich in tannins such as ellagitannin and punicalagin. These latter exhibit inhibitory activity by binding to the active site of the urease or by modulating its activity via their aggregation properties.

Additionnally,-in a recent study by **Al-Rooqi et *al.* (2023)**, the structure-activity relationship was studied. For example, flavones such as quercetin, kaempferol, and myricetin, which are components of *P.granatum*, showed competitive inhibition of urease due to their hydroxyl groups. Furthermore, an electron donation on their benzene ring at positions m- and p- showed an improvement in the inhibitory activity.

Also, **Biglar et *al.* (2021)** reported that the hydroxyl group in the 3,5,7-trihydroxy-4H-chromen-4-one ring, and the hydroxyl group at the fourth position of the catechol ring on quercetin, play an important role in the inhibition of urease.

III.3. Molecular docking

The structural interaction between a ligand and a receptor can be predicted using molecular docking. As per the docking law of discovery studio, a reduced energy value signified that the docking system of the receptor and ligand was more stable **(Zhang et *al*., 2015)**.

In this study, we have chosen two phenolic compounds with high concentrations in *Punica granatum* (pomegranate): punicalagin and ellagic acid. These compounds are highly bioactive and provide numerous human health benefit. Punicalagin, a large polyphenol classified as an ellagitannin, is a potent antioxidant that reduces oxidative stress, inflammation, and has anticancer properties. It also supports cardiovascular health by lowering blood pressure and inhibiting LDL cholesterol oxidation. Ellagic acid, a naturally occurring polyphenol, exhibits strong antioxidant and anti-inflammatory properties, inhibits cancer cell proliferation, and induces apoptosis. Both compounds have demonstrated antimicrobial and neuroprotective effects, making them valuable in promoting overall health and preventing chronic diseases **(Rummun et *al*., 2013 ; Fouad et *al*., 2016 ; Rozadi et *al*., 2022 ; Sharifi-Rad et *al*., 2022 ; Alalawi et *al*., 2023 ; Zhizhou et *al*., 2024)**.

To identify the ligand-binding mechanism and pinpoint the amino acids in the ligand and receptor binding sites, molecular docking was carried out in the XO and urease ligand-binding pocket.

The primary target crystal structure utilized for *in vitro,* testing was the bovine XO co-crystallized with guanine (PDB ID 3NVW), which has a 90% overall sequence homology with human xanthine oxidase. To verify the effectiveness of the docking techniques, the docked complexes were aligned and superimposed on native co-crystallized XO protein complex.

The bovine xanthine oxidase with PDB ID: 3NVW is an enzyme with 1 254 amino acids. It is an enzyme with a molecular weight of 280 kDa. The active site of 3NVW is a small cavity, and from a structural point of view, it is divided into two sections. One section impart specificity to the ligand (substrate or inhibitor), and the second is preserved to the cofactors. The docking site was centered at the position of the docked ligand which is downloaded with the protein. The docked complexes' RMSD value, when compared to the reference co-crystallized protein complexes that were aligned, was 2.7.

This shows that the docking method utilized in this study is acceptable and can accurately anticipate the poses of additional molecules, as has already been confirmed by **(Ramírez & Caballero,2018)**.

When the binding affinity of the two phenolic compounds was compared to allupurinol (ΔG of -8.10 kcal/mol) **(Table V)**, it was found that punicalagin showed the best binding affinity with a ΔG of -11.32 kcal/mol. However, Ellagic acid showed a little higher energy (-7.49 kcal/mol). Table VII shows the inhibition constants of the docked targeted protein receptors with the selected compounds. Inhibition constant is directly proportional to binding energy. We found a decrease in inhibition constant of the selected compounds with a simultaneous decrease in the binding energy. Thus, the xanthine oxidase inhibitory activity of punicalagin was found to be higher compared to allopurinol **(Umamaheswari et *al*.,2011)**.

Table V: Docking score and Interactions of ligands Docked to xanthine oxidase.

Molecule	**Docking score (kcal/mol)**	**Ki**	**Interactions**		
			Interaction type	Amino acid	Distance (Å)
Allopurinol	-8.10	1.16 uM	H-bond pi-alkyl pi-pi stacked pi sigma C-H bonds	Glu802 Arg880 Ala1079 Leu873 Phe914 Ser876	1.98 3.05 4.48/ 5.50 5.38 3.89/4.04 3.94 3.33
Ellagic Acide	-7.49	3.26 uM	H-bond Alkyl Sulfur-x	Ser1082 Gln1194 Thr1077 Arg912 Ala1078 Met1038	2.90 1.84/1.99 2.08 5.20 4.84 2.75
Punicallagin	-11.32	5.00 nM	Hbond Pi alkyl Amide pi stacked C-H bonds	Arg912 Glu1194 The1083 Ser1082 Val1081 Met1038 Ser1080 Ala1078	2.57 2.25 2.02 3.35 2.20 4.67 4.48 2.98

According to **Azani et *al*. (2011),** hydrogen bonds and n-n hydrophobic interactions between the antigout molecule and the receptor's active regions are typically thought to mediate the biological activity of these drugs.

BIOVIA Discovery Studio Visualizer 4.1 was used to analyze and visualize the interaction patterns of target XO proteins and chemicals. Allopurinol binds to XO through strong hydrogen bonds formed by Glu802, Arg 880 at a distance of 1.98 and 3.05 Å respectively and by hydrophobic contacts formed by Ala 1078, Leu873 and Phe914 (spaced by 3.98 to 5.50A) and a covalent carbon hydrogen bond with Ser876 (5.38A°).

Punicalagin takes on a particular orientation at the binding site of XO. It interacts with XO by covalent C-H bonds (Ala1078), hydrophobic interactions (Met1038 and Ser1080), five H-bonds (Arg912 (2.57 A°), Gln1194 (2.25 A°), Thr1083 (2.02 A°), Ser1082 (3.35 A°), and Val1081 (2.20 A°) **(Figure 12).** Ellagic acid formed four hydrogen interactions with XO at Ser1082 (2.90A°), Gln1194 (1.84 and 1.99A°), and Thr1077 (2.08A°). Additionally, it exhibited hydrophobic interactions with Arg912 and Ala1078, as well as a sulfur bond with Met1078 **(Figure 13).**

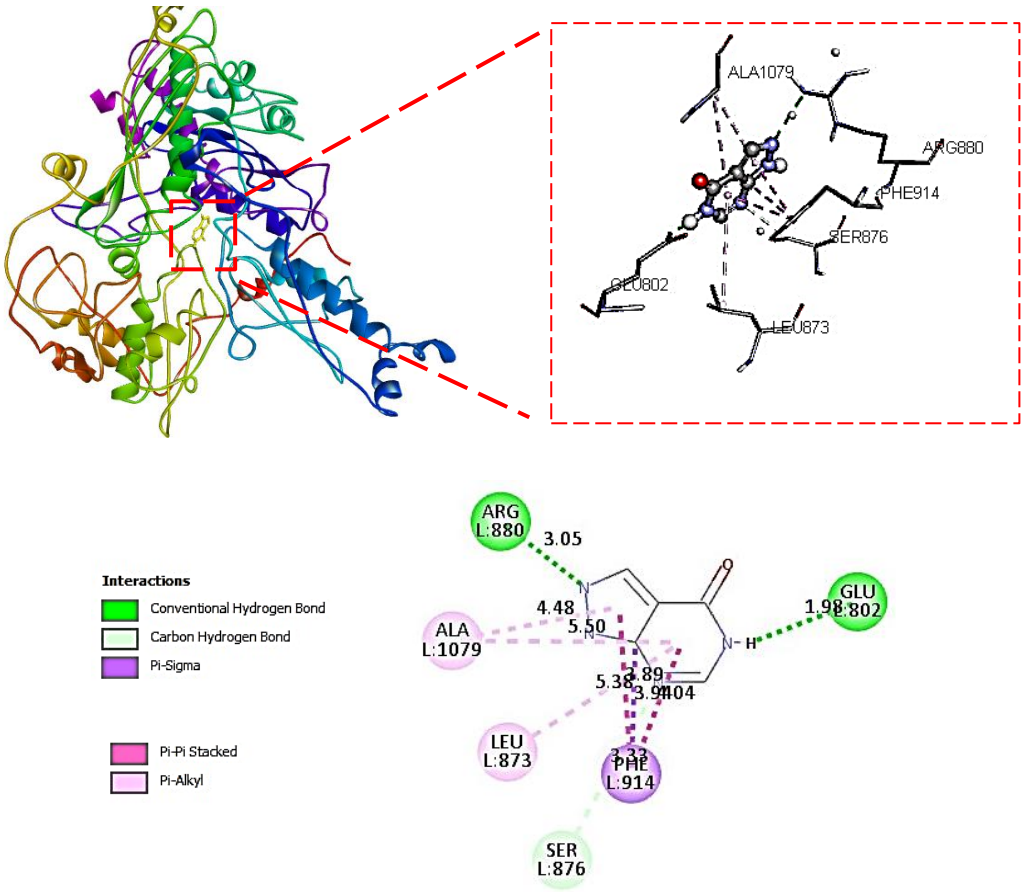

Figure 11: Molecular docking. Three-dimensional (above), two-dimensional (below) ligand interaction diagrams of Allopurinol with xanthine oxidase (3NVW).

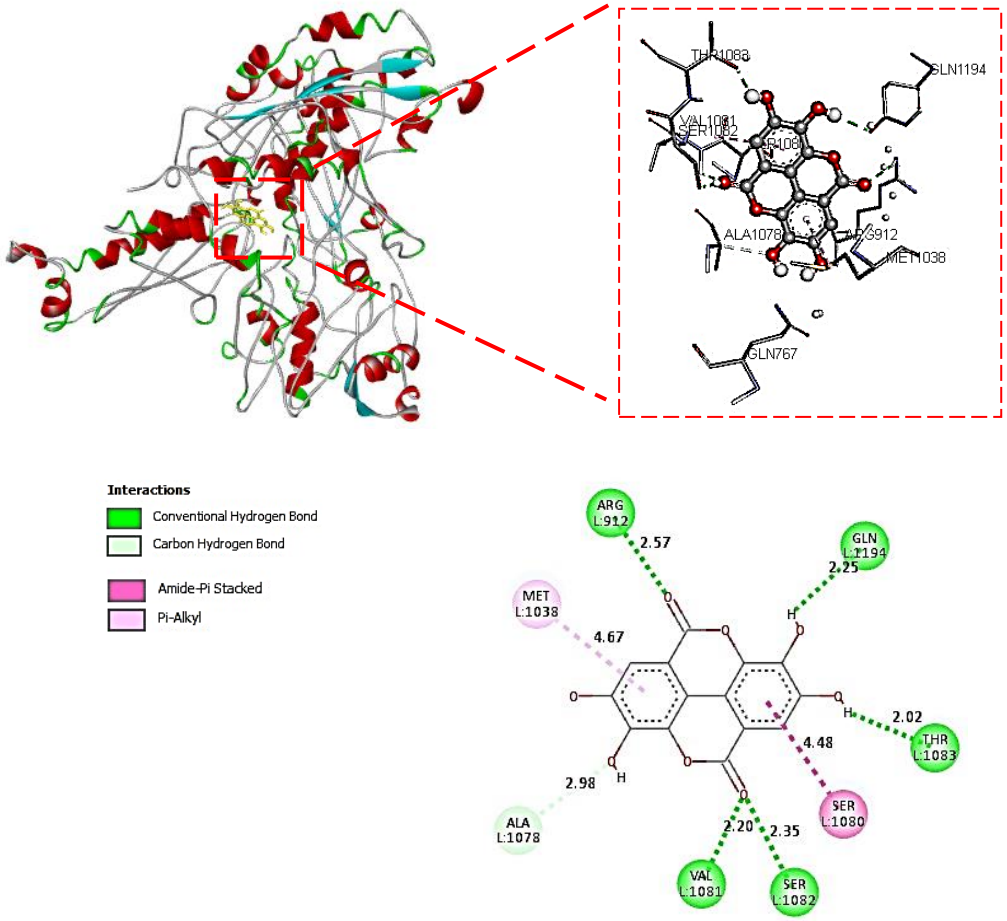

Figure 12: Molecular docking. Three-dimensional (above), two-dimensional (below) ligand interaction diagrams of Punicalagin with xanthine oxidase (3NVW).

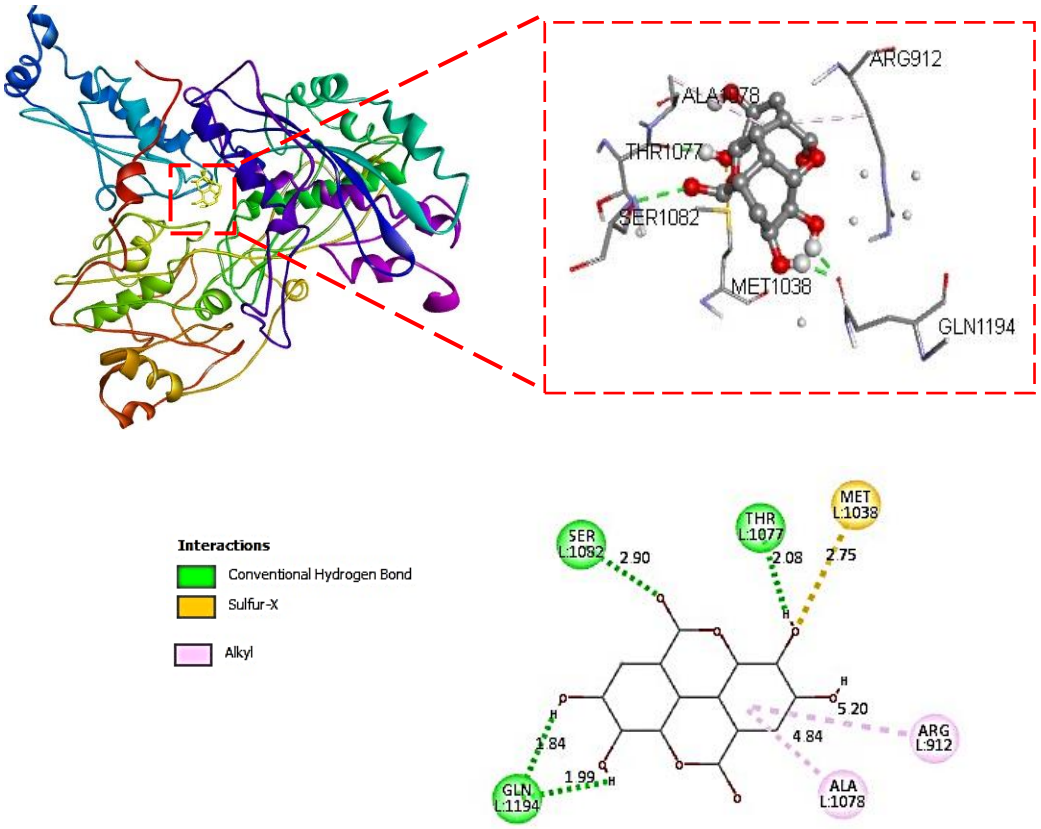

Figure 13: Molecular docking. Three-dimensional (above), two-dimensional (below) ligand interaction diagrams of ellagic acid with xanthine oxidase (3NVW).

In 2020, Adachi et al., published a study demonstrating the ability of ellagic acid to lower uric acid levels. They found that ellagic acid successfully reduced uric acid synthesis in AML12 hepatocytes and prevented the increase in plasma uric acid levels in the experimental group. Qing-qing Han's et *al.* (2024) study investigated the use of punicalagin as an inhibitor on a mouse model of hyperuricemia. The study indicated that punicalagin significantly improved hyperuricemia in the animals by restoring kidney and intestinal function. Punicalagin has the potential to improve the production of uric acid transporters in the kidney and intestine by inhibiting the activation of inflammatory signaling pathways. In addition, punicalagin was discovered to improve the imbalance of gut microbiota and the abnormality in renal glycometabolism in the mice model with hyperuricemia. This study discovered that punicalagin has the ability to lower uric acid levels in individuals with hyperuricemia. It also examined the underlying mechanisms involving the kidneys and intestines.

These findings suggest that punicalagin could be a promising nutraceutical for treating high uric acid levels in clinical trials. This is particularly noteworthy due to its high safety margin and numerous reports of its effectiveness in humans.

For urease, the interaction between the same compounds and specific binding sites of urease through hydrogen bonding, metal/ion contact with Ni ions, and hydrophobic interactions was evaluated and compared to boric acid as standard molecule.

Table VI and Figure 14,15,16 summarize all details related to the docking study of punicalagin, ellagic acid and boric acid in the binding site of urease. Upon comparing the binding affinity of the two phenolic compounds to boric acid (with a ΔG of -6.16 kcal/mol), it was observed that punicalagin had the best binding affinity with a ΔG of -9.00 kcal/mol, followed by ellagic acid (ΔG = -7.53 kcal/mol). Boric acid has the higher binding energy (-6.16 kcal/mol). Ki was equal for binding energy and was 30.29 µM, 254.59 nM and 3.02 µM for boric acid, punicalagin and ellagic acid respectively.

Table VI: Docking score and Interactions of Ligands Docked to Urease.

Molecule	**Docking score (kcal/mol)**	**Ki**	**Interactions**		
			Interaction type	Amino acid	Distance (Å)
Boric Acid	-6.16	30.29 uM	H bond C-H bonds	Tyr544 His545 Ile518 Thr520 Gly552 His519	2.62 3.96 2.69 1.95 3.27 3.37
Ellagic acid	-7.53	3.02 uM	H bond Alkyl pi alkyl C-H bonds	Arg609 Asp494 Arg439 Ala440 His492 Ala636 Met637 His593 Ala440 His409	2.02 1.93 2.87/1.78 2.27 2.34 4.92 4.82 4.15 3.20 3.44
Punicalagin	-9.00	254.59 nM	H bond pi alkyl pi sigma pi pi stacked pi cation pi anion	His492 Asp633 Ala440 Ala636 Ala440 His593 Asp494 His593	1.84 2.95 5.06 / 4.61/4.44 4.27 / 4.83 3.90 5.06 4.07 3.75

The computational molecular docking results indicate that punicalagin forms hydrogen bonds with the Ni901 atom at distances of 2.32 and 2.73A°. Additionally, punicalagin forms hydrogen bonds with His492 and Asp633 at distances of 1.84 and 2.95, respectively. Punicalagin is also flanked by four other amino acids, specifically Ala440 (5.06, 4.61, 4.44A°) and Ala636 (4.27, 4.83A°), as well as Asp494 (3.90A°), His593 (5.06A°), Asp494 (4.07 A°) and His593 (3.75 A°) forming hydrophobic interaction.

The precise positioning of punicalagin with urease is of utmost significance as it allows us to get a deeper understanding of the interactions between proteins and ligands. This knowledge can provide valuable insights into the functionality and effectiveness of punicalagin and other ligands that have the potential to be used as therapeutic agents. The literature confirms our docking results based on the existence of these functions **(Saeed et *al*.,2017)**.

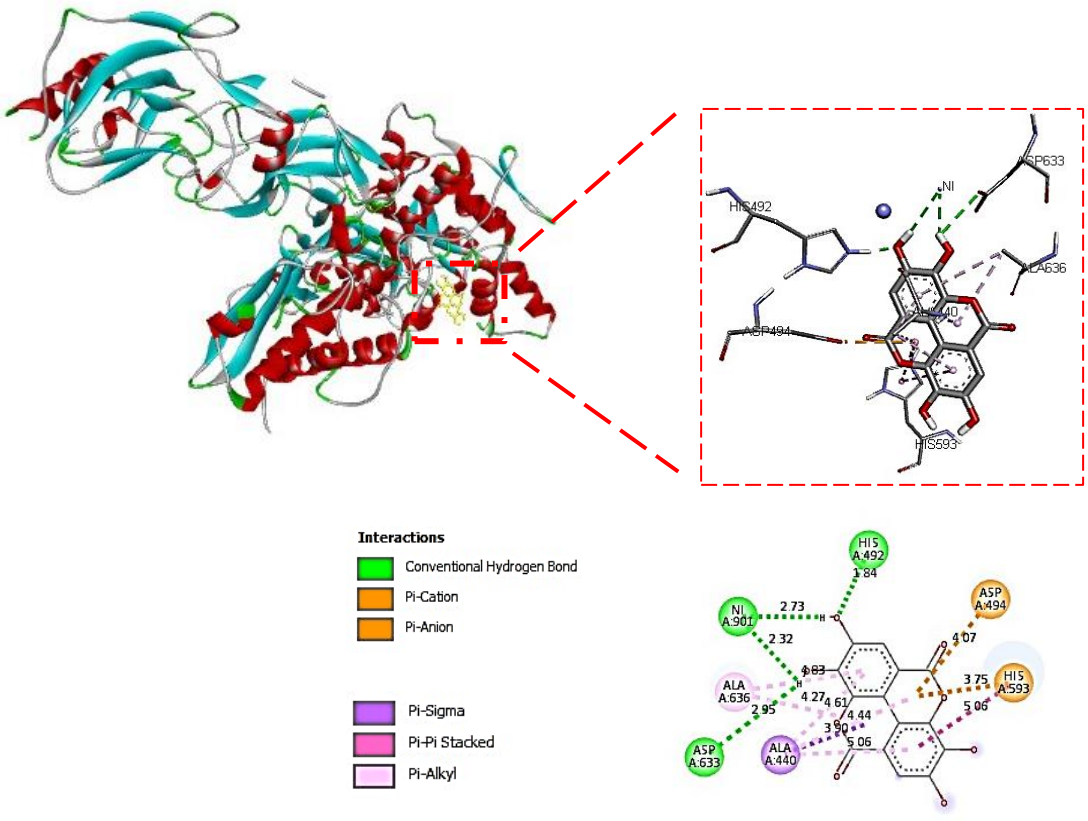

Figure 14: Molecular docking. Three-dimensional (above), two-dimensional (below) ligand interaction diagrams of Punicalagin with urease (4H9M).

Ellagic acid interact by two hydrogen bonds with Ni901and Ni902 atoms, and is surrounded by five Arg609 (2.02 A°), Asp494 (1.93 A°), Arg439 (2.87 and 1.78 A°), Ala440 (2.27 A°) and His492 (2.34 A°) throw hydrogen bonds **(figure 18)**. Apart from these residues, it has also interacted with Ala636, Met637 and His593 forming alkyl bonds (4.92, 4.82 and 4.15A°), and covalent C-H bond with Ala440 and His409 residues (3.20 and 3.44A° respectively). However, we noticed that boric acid didn't interact with Ni atoms, but surrounded by Tyr544, His545, Ile518 and Thr520 throw hydrogen (3.96 to 1.95A°) bonds, Gly552 and His519 throw C-H bonds. In fact, punicalagin and ellagic acid interact with one of the critical residues of the active site namely Asp633 for punicalagin and Arg439 for ellagic acid which could explain their binding.

In fact, despite ellagic acid having more hydrogen bonds with the protein compared to punicalagin, punicalagin showed better binding energy. Weak intermolecular interactions, such as hydrogen bonding and hydrophobic interactions, play a crucial role in stabilizing ligands within protein structures.

According to Patil et *al*. (2010), adding more hydrophobic atoms to the drug-target interface can boost the drug's biological activity by increasing the binding affinity. Combining hydrophobic interactions with hydrogen bonding at the binding site can enhance both the binding affinity and the drug's effectiveness.

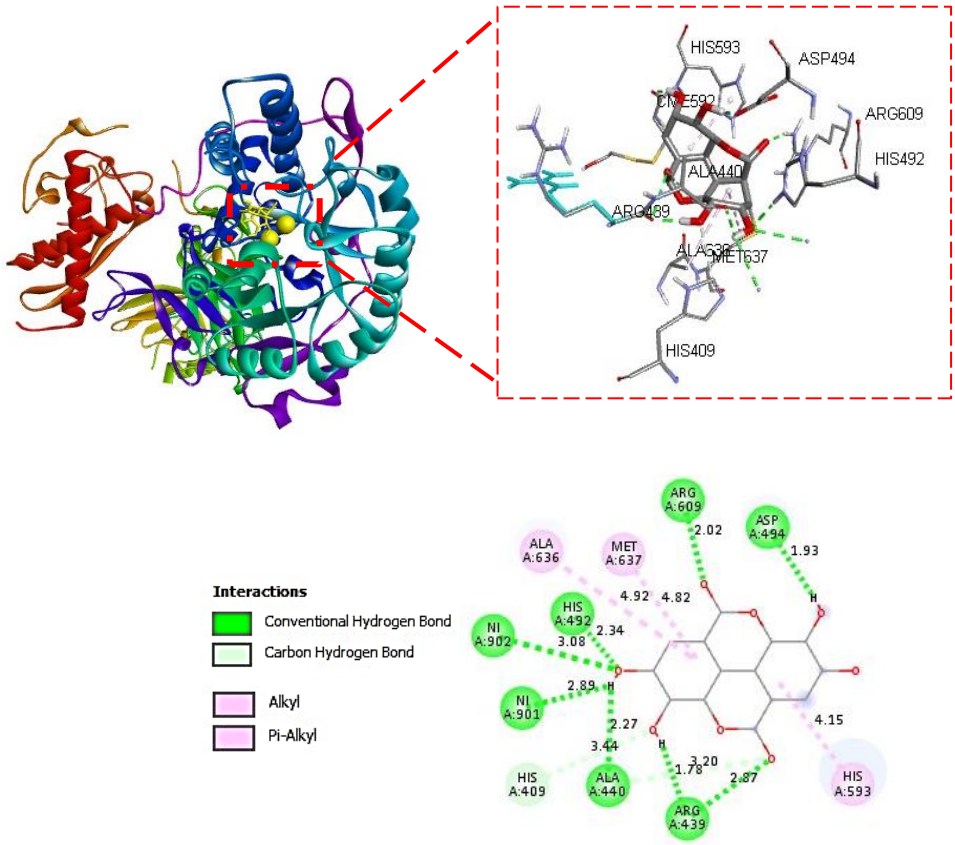

Figure 15: Molecular docking. Three-dimensional (above), two-dimensional (below) ligand interaction diagrams of Ellagic acid with urease (4H9M)

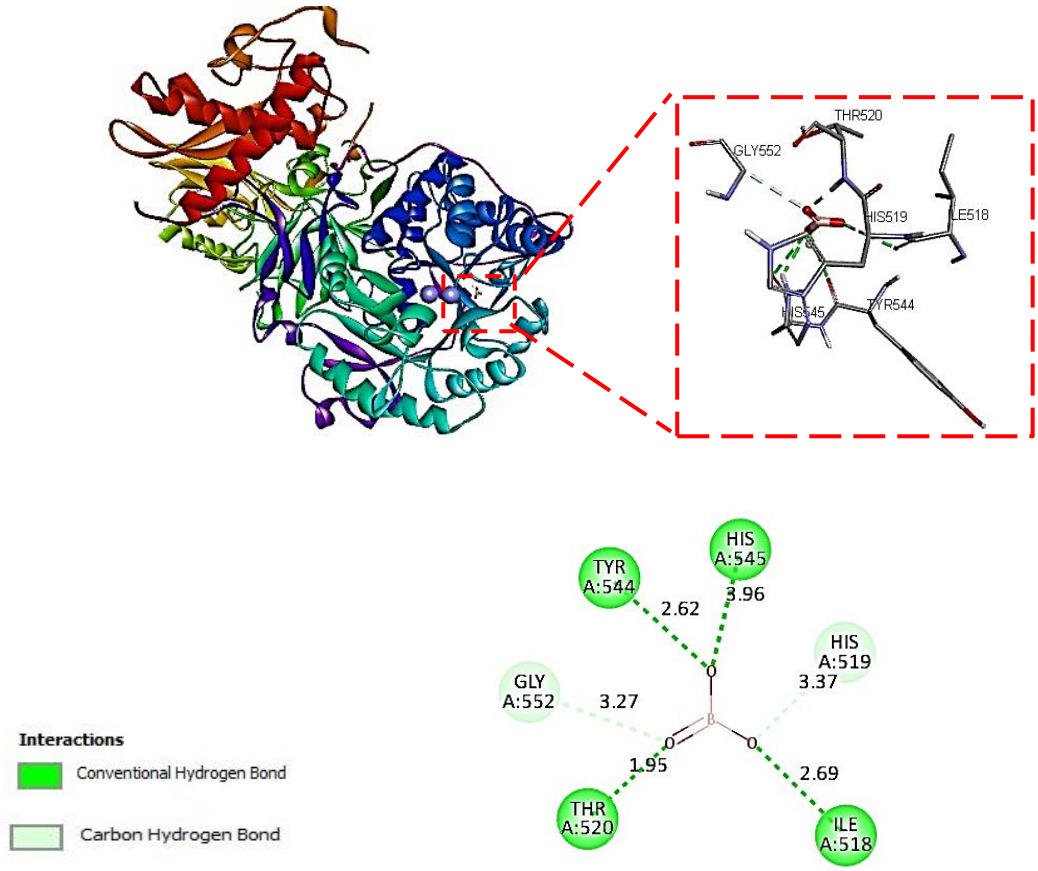

Figure 16: Molecular docking. Three-dimensional (above), two-dimensional (below) ligand interaction diagrams of boric acid with urease (4H9M)

III..4. Anti-urolithiasic activity of *Punica granatum* extracts

III.4.1. Litholytic activity of *Punica granatum* extracts against calcium oxalate monohydrate (COM) crystals

The impact of *Punica granatum* peel and flower extracts on the dissolution and concentration decrease of calcium oxalate monohydrate (COM) crystals was monitored using a turbidity assay. A concentration of 200 µg/mL and 500 µg/mL were implemented for the COM crystals (figure 20 and 21).

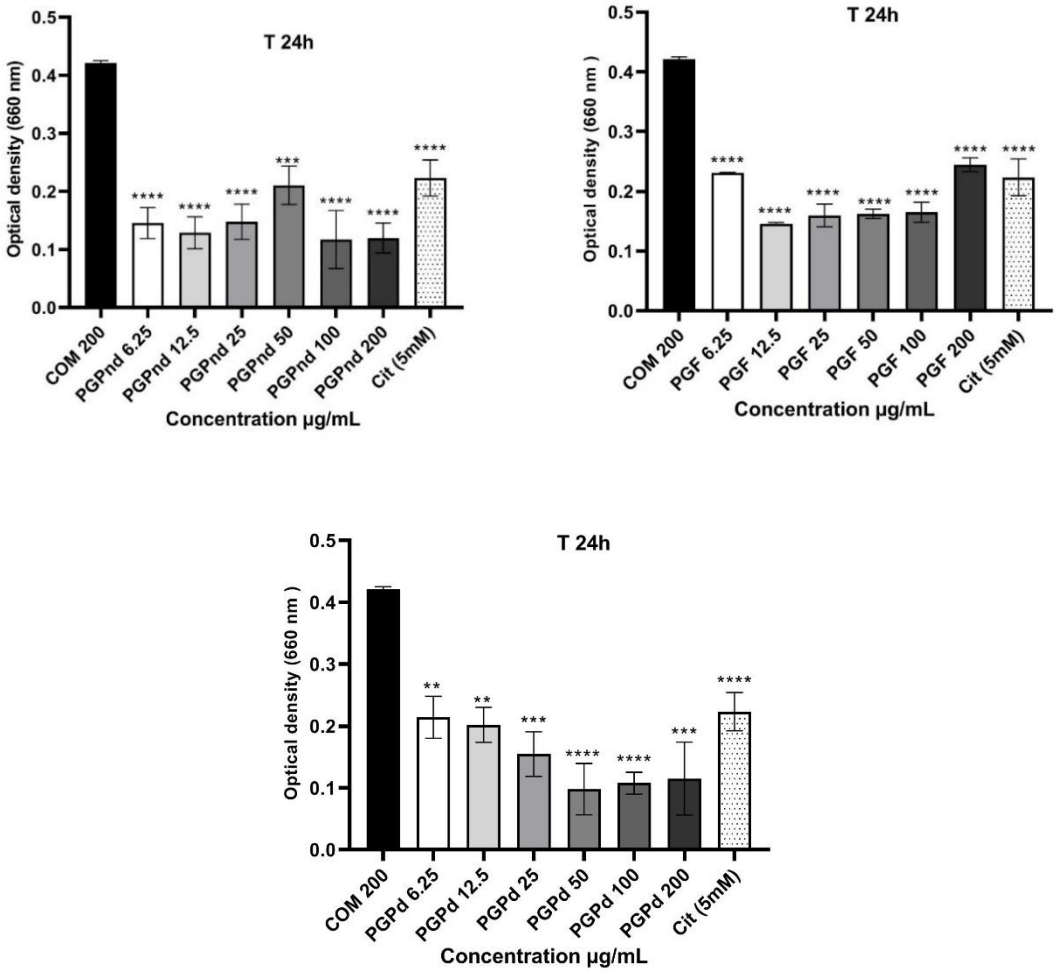

Figure 17: Effect of *Punica granatum* extracts and citrate on COM crystal at 200µg/mL optical density (OD) for 24h. PGF: aqueous flower extract, PGPnd: ethanolic non-delipidated peel extract, PGPd: ethanolic delipidated peel extract. Cit (5Mm): Citrate (5Mm). All experiments are mean ± SD of triplicate. Significance ($^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$ $^{****}p < 0.0001$) compared to COM 200µg/mL is expressed by one way ANOVA test.

Optical density measurements were taken to estimate the change in the concentration of COM for the different experimental groups. Both delipidated and non-delipidated ethanolic peel extract, as well as the aqueous flower extract, were assessed. The assay was conducted at various time intervals over a period of 24 hours. The results showed that the COM control had the highest optical density for both concentrations tested (200 µg/mL and 500 µg/mL) indicating a high crystal concentration.

The results obtained at 24h, show that citrate (5 mM) like *Punica granatum* extracts reduced the optical density in a very highly significant manner for the tested concentrations to COM ($p < 0.0001$).

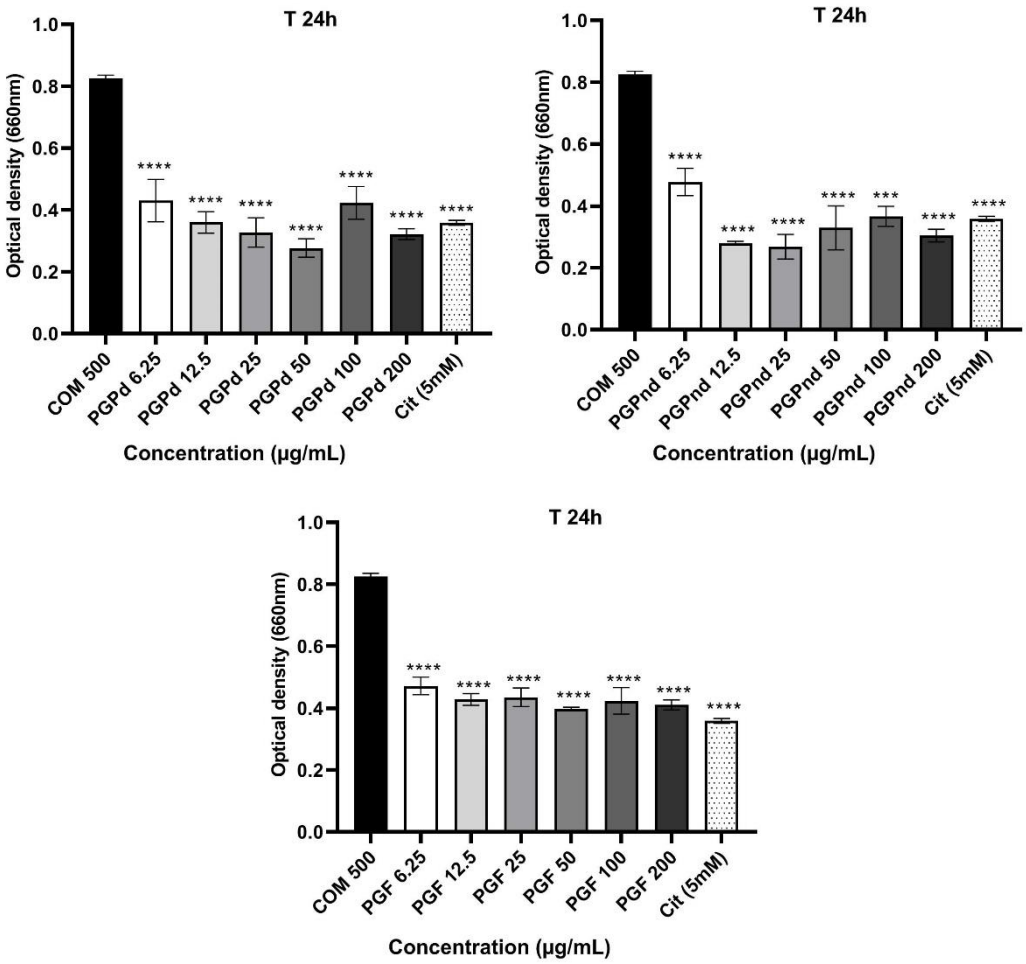

Figure 18: Effect of *Punica granatum* extracts and citrate on COM crystal at 500µg/mL optical density (OD) for 24h. PGF: aqueous flower extract, PGPnd: ethanolic non-delipidated peel extract, PGPd: ethanolic delipidated peel extract. Cit (5Mm): Citrate (5Mm). All experiments are mean ± SD of triplicate. Significance ($^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$ $^{****}p < 0.0001$) compared to COM 500µg/mL is expressed by one way ANOVA test.

The results obtained for COM at a concentration of 500 µg/mL showed that all extracts, exhibited a highly significant effect ($p < 0.0001$) in reducing turbidity at concentrations ranging from 6.25 to 200 µg/mL compared with control group.

On the other hand, except the ethanolic of PGP delipidated extract which presents a dose-response variation profile, in reducing turbidity at concentrations 200 µg/mL, after 24 hours of incubation, the aqueous flower extract and ethanolic of PGP non-delipidated extract showed a very highly significant effect ($p < 0.0001$) at all tested concentrations.

The study by **Cheraft –Bahloul et *al*. (2017)** assessed the dissolution effect of an ethanolic delipidated of *Pistacia lentiscus* extract on COM crystals. Their results showed that the lowest concentration (35 µg/mL) was the most effective **(Cheraft – Bahloul et *al*., 2017).**

Kachkoul et *al*. (2019) used an aqueous *Arbutus unedo* L., extract that demonstrated even better results than the ethanolic extract. In our study, the aqueous flower extract of *Punica granatum* showed highly significant results for all concentrations tested for both COM 200 µg/mL and COM 500 µg/mL. Furthermore, **Kachkoul et *al*.** (**2019)** research, conducted using an alternative approach, also demonstrated a dissolution rate that is dependent on the concentration. This finding was particularly noteworthy when compared to the citrate standard. The acquired results for both COM concentrations were consistent with the results reported for the ethanol peel extract.

Moreover, **El Habbani et *al*. (2021)** worked on an aqueous extract of *O. ficus-indica* flower and their results were better than citrate used as a standard. Our results concurred with their study. These conclusions indicates that our extracts exhibit a similar behavior to those studied by other researchers.

The effectiveness of the extracts used in this study depends on their ability to dissolve calcium oxalate stones. A recent review conducted by Maphetu et *al*. (2022) has confirmed that both the aqueous flower extract and the ethanolic peel extract of *Punica garantum* contain a variety of bioactive compounds, including flavonoids, tannins, saponins, alkaloids, quinones, cardiac glycosides, terpenoids, phenols, coumarins, and steroids **(Maphetu et *al*., 2022)**. El Habbani et *al*. (2021), suggested that these compounds have litholytic properties and can interact with the calcium oxalate crystals. This interaction involves the formation of a complex via both hydrogen and hydrophilic bonds, making the crystals more soluble.

III.4.2. Effects of *Punica granatum* L. flower extract against calcium oxalate monohydrate-induced alteration on sperm motility

In this study, we used the CASA system to examine the kinetics of sperm motility parameters over a period of 24H. This allowed us to analyze the movement patterns of the spermatozoa cells. The key parameters we focused on were Straight-line velocity (VSL), Curvilinear velocity (VCL), and Average path velocity (VAP), as they provide the most accurate insights into the behavior of the sperm cells. The definition of each of these parameters as shown in **Table V** has been determined by the World Health Organization and was recently reported by **Hook & Fisher (2020) (Annexe II).**

The results shown in figure 22 indicated a significant decrease in all parameters of sperm motility (VSL, VCL, VAP) for the group treated with a COM at a concentration of 500 µg/mL compared to the control group at T1 and T 24h. Citrate (5 Mm), used as a standard, was found to be toxic to sperm as it significantly decreased sperm movement as this could be due to the low pH.

It's important to point out that only *Punica granatum* flower extract was tested, in this study. The incubation of intoxicated sperm (COM 500 µg/ml) with concentrations of *Punica granatum* flower extract (50-1000 µg/mL), induced the restoration of motility parameters for all concentrations, compared to the positive control (COM 500µg/Ml) ($p < 0.0001$), and the more effective effect was with the lowest concentrations (50 and 100µg/mL) with values higher than the negative control, at T1. Furthermore, this restoration of motility was maintained after 24 hours, where the values of VSL, VCL and VAP in the treated groups with the different concentrations of the PGF extract were statistically similar to those of the control.

The spermatozoa cells, are increasingly used in research for various *ex vivo* studies as it is an abundant source of cell material that is readily available **(Vollmer et *al*., 2019; Moretti et *al*., 2023)**. Spermatozoa are highly differentiated cells with specific characteristics namely motility **(World Health Organization, 2010; Vollmer et *al*., 2019; Moretti et *al*., 2023)**, a very low level of transcription and translation **(Baker & Aitken, 2009; Jodar et al., 2016; Vollmer et *al*., 2019; Moretti et *al*., 2023)**, lack of DNA repair activity **(Setti et *al*., 2021; Moretti et *al*., 2023),** but also a remarkable lack of intracellular antioxidant activity with a low ability to repair damage caused by oxidative stress **(Aitken et *al*., 2022; Moretti et *al*., 2023)**.

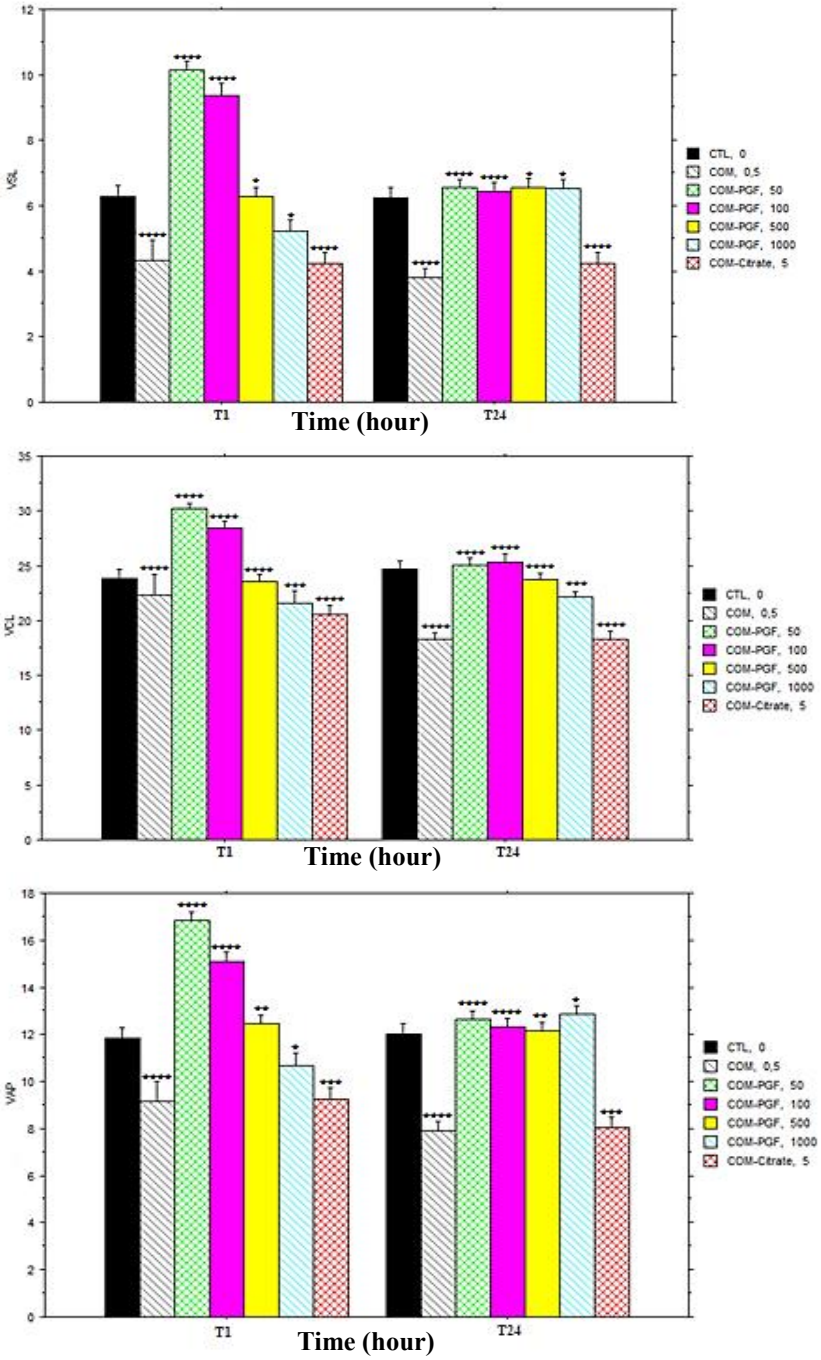

Figure 19: Effects of *Punica granatum* L. extract and Citrate against COM at 500 µg/mL, induced alteration on sperm motility: A: of Straight-line velocity (VSL). B: Curvilinear velocity (VCL) and C: Average path velocity (VAP), for 1h and 24h. PGF: aqueous flower extract. Cit: Citrate (5Mm). All experiments are mean ± SEM of triplicate. Significance ($^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$ $^{****}p < 0.0001$) compared to control group is expressed by the F test of variance equality.

Their sensitivity to external conditions makes them an ideal tool for toxicity testing **(Moretti et *al*., 2023).** Moreover, these cells have become a toxicity monitor for several xenobiotics, including natural substances **(Shaliutina et *al*., 2021; Moretti et *al*., 2023)**.

Different toxic agents were studied using spermatozoa as a model in the context of several diseases such as certain herbicides **(Tan et *al*., 2016; Moretti et *al*., 2023)**, heavy metals **(Chen et *al*., 2022)**, nanoparticles **(Moretti et *al*., 2013; Santonastaso et *al*., 2020)** and some drugs **(Xu et *al*., 2013; Ali Banihani & Al khawalde, 2019; Moretti et *al*., 2023).** In relation to kidney disease, sperm were used as a model for a study that was conducted by **Vollmer et *al*. (2019)** on the toxicity of uremic substances in the case of uremia (**Vollmer et *al*., 2019)**. Until now, no studies on using COM crystals on spermatozoa have been published. Additionally, only a few studies have utilized pomegranate in sperm studies.

However, **Tuck et *al*., (2010)** implemented the use of *Punica granatum* juice to improve sperm production quality, showing a significant improvement in spermatozoa motility in rats in a dose-dependent manner, which aligns with our results. **Fedder et *al*. (2014)** also supported similar results in male adult participants.

Oxidative stress is the major factor affecting sperm cell motility, as these cells are sensitive to reactive oxygen species (ROS). Due to their high content of polyunsaturated fatty acids in the membrane, this makes them more susceptible to lipid peroxidation and loss of cell mobility **(Khan, 2011; Ghadimi et *al*., 2024**).

According to **Tremellen (2008)**, two pathways could alter sperm quality. Either the ROS act as free radicals, damaging the cell membrane and lowering spermatozoa motility, or the damage of cell DNA. This imbalanced state could be restored by the presence of antioxidants. *Punica granatum* is rich in antioxidant agents that exhibit antioxidant activity based on their structure. As per **Madrigal-Carballo et *al*. (2009)**, polyphenolic molecules act as reducing agents, providing hydrogen in a redox reaction through one of their hydroxyl groups. *Punica granatum* extracts are rich in bioactive compounds such as gallic acid, ellagic acid, punicalin, and punicalagin.

A recent study conducted by **Zhang et *al*. (2024)** used punicalagin to improve sperm motility, and they proved that punicalagin increased sperm motility, especially at lower concentrations. This effect is also noticed in our extract. This effect is due to the decrease of ROS (**Zhang et *al*., 2024)**.

Conversely, **Ghadimi et *al*. (2024)** studied the effect of gallic acid on sperm motility and quality, and they obtained significant results as the beneficial antioxidant effect reduced oxidative stress and markedly decreased malondialdehyde (MDA) levels (**Ghadimi et *al*., 2024)**.

All these results confirm the improvement of motility by the presence of antioxidants. According to the literature, *Punica granatum* flower extract is very rich in the above-mentioned bioactive agents, which aligns with the results obtained, showing that the flower extract significantly improved sperm quality even in the presence of a toxic agent such as COM crystals.

Conclusion and perspectives

Conclusion and perspectives

The objective of this study was to determine the efficacy of *Punica granatum* extract, as an anti-urolithiasic agent by applying it to various experimental study models.

The results obtained from the Ferric Reducing Antioxidant Power assay showed a high antioxidant power in both the aqueous flower extract and the ethanolic non-delpidated peel extract.

The aqueous flower extract exhibited better xanthine oxidase inhibitory activity with an IC_{50} = 38.11±7.835 µg/mL compared to other extracts, although it was less effective than allopurinol, the standard molecule. In the urease inhibitory assay, all extracts produced better results than the boric acid standard, but the ethanolic non-delpidated peel extract showed the best result with an IC_{50} =32.61±0.625 µg/mL.

To gain more insight into these findings, we performed molecular docking analysis on key phenolic compounds found in *P. granatum* extracts, specifically ellagic acid and punicalagin to study their interaction with both enzymes used in the *in vitro* model. The result indicated the effectiveness of punicalagin on inhibiting xanthine oxidase and urease by demonstrating the weakest binding energy (-11.32 kcal/mol) and (-9.00 kcal/mol) respectively, and a good molecular interactions with these enzymes.

Moreover, the litholytic test of *P. granatum* extracts revealed that all concentrations tested were highly effective at 24 hours, similar to the standard molecule citrate for both COM concentration (200 and 500µg/mL). In the *semi in vivo* model applied to spermatozoa, the *Punica* aqueous flower extract showed a highly significant improvement in the motility parameters, including VSL, VCL, and VAP, at different concentrations over time.

In future studies, it would be interesting to investigate the enzymes inhibition mode of each extract. Additionally, using separating methods such as HPLC to determine the composition of the plant extracts would be useful for molecular docking to test several other components for efficiency. Also studying the ADME and toxicity of the molecules. As for the spermatozoa study model, it would be interesting to study the antioxidant status, DNA integrity. These studies will advance scientific research and improve the understanding and study of urolithiasis, and the development of new natural treatments.

Bibliographic references

- Adachi, S., S asaki K., Kondo S., Komatsu W., Yoshizawa F., Isoda H., et al. (2020). Antihyperuricemic Effect of Urolithin A in Cultured Hepatocytes and Model Mice. *Molecules*. 25, 5136. https://doi.org/ 10.3390/molecules25215136
- Aggarwal, K. P., Narula, S., Kakkar, M., & Tandon, C. (2013). Nephrolithiasis: Molecular Mechanism of Renal Stone Formation and the Critical Role Played by Modulators. *BioMed Research International*, *2013*, 1–21. https://doi.org/10.1155/2013/292953
- Agu, P. C., Afiukwa, C. A., Orji, O. U., Ezeh, E. M., Ofoke, I. H., Ogbu, C. O., Ugwuja, E. I., & Aja, P. M. (2023). Molecular docking as a tool for the discovery of molecular targets of nutraceuticals in diseases management. *Scientific Reports*, *13*(1), 13398. https://doi.org/10.1038/s41598-023-40160-2
- Aitken, R. J., Bromfield, E. G., & Gibb, Z. (2022). Oxidative stress and reproductive function: The impact of oxidative stress on reproduction: A focus on gametogenesis and fertilization. *Reproduction*, *164*(6), F79-F94.
- Alalawi, S., Albalawi, F., Ramji, DP. (2023). The Role of Punicalagin and Its Metabolites in Atherosclerosis and Risk Factors Associated with the Disease. International Journal of Molecular Sciences. 9;24(10):8476. doi: 10.3390/ijms24108476
- Alelign, T., & Petros, B. (2018). Kidney Stone Disease: An Update on Current Concepts. *Advances in Urology*, *2018*, 1–12. https://doi.org/10.1155/2018/3068365
- Ali Banihani, S., & Al-khawalde, A. A. A. (2019). Omeprazole does not alter human sperm motility, viability or DNA integrity in vitro. *Andrologia*, *51*(6), e13260.
- Al-Rooqi, M. M., Mughal, E. U., Raja, Q. A., Hussein, E. M., Naeem, N., Sadiq, A., et al. A. (2023). Flavonoids and related privileged scaffolds as potential urease inhibitors: a review. *RSC advances*, *13*(5), 3210-3233.
- Amr A. Fouad, Hatem O. Qutub, Walid N. Al-Melhim, Punicalagin alleviates hepatotoxicity in rats challenged with cyclophosphamide, *Environmental Toxicology and Pharmacology*, 45, 2016, Pages 158-162, https://doi.org/10.1016/j.etap.2016.05.031
- Ardekani, M. R. S., Hajimahmoodi, M., Oveisi, M. R., Sadeghi, N., Jannat, B., Ranjbar, A. M., Gholam, N., & Moridi, T. (2011). *Comparative Antioxidant Activity and Total Flavonoid Content of Persian Pomegranate (Punica granatum L.) Cultivars*.
- Atmani, D., Chaher, N., Atmani, D., Berboucha, M., Debbache, N., & Boudaoud, H. (2009). Flavonoids in human health: from structure to biological activity. *Current Nutrition & Food Science*, *5*(4), 225-237.
- Bai, S., Bharti, P., Seasotiya, L., Malik, A., & Dalal, S. (2015). In vitro screening and evaluation of some Indian medicinal plants for their potential to inhibit Jack bean and bacterial ureases causing urinary infections. *Pharmaceutical biology*, *53*(3), 326-333.
- Baker, M. A., & Aitken, R. J. (2009). Proteomic insights into spermatozoa: critiques, comments and concerns. *Expert review of proteomics*, *6*(6), 691-705.Bashir, S., and Gilani, AH. (2011). Antiurolithic effect of berberine is mediated through multiple pathways. *European Journal of Pharmacology*. 651(1-3):168-175. https://doi.org/10.1016/j.ejphar.2010.10.076
- Barr-Beare, E., Saxena, V., Hilt, E. E., Thomas-White, K., Schober, M., Li, B., Becknell, B., Hains, D. S., Wolfe, A. J., & Schwaderer, A. L. (2015). The Interaction between Enterobacteriaceae and Calcium Oxalate Deposits. *PLOS ONE*, *10*(10), e0139575. https://doi.org/10.1371/journal.pone.0139575
- Benchagra, L., Berrougui, H., Islam, M. O., Ramchoun, M., Boulbaroud, S., Hajjaji, A., Fulop, T., Ferretti, G., & Khalil, A. (2021). Antioxidant Effect of Moroccan Pomegranate (Punica granatum L. Sefri Variety) Extracts Rich in Punicalagin against the Oxidative Stress Process. *Foods*, *10*(9), 2219. https://doi.org/10.3390/foods10092219
- Bichler, K.-H., Eipper, E., Naber, K., Braun, V., Zimmermann, R., & Lahme, S. (2002). Urinary infection stones. *International Journal of Antimicrobial Agents*, *19*(6), 488–498. https://doi.org/10.1016/S0924-8579(02)00088-2

- Bigelow, M. W., Wiessner, J. H., Kleinman, J. G., & Mandel, N. S. (1996). Calcium Oxalate-Crystal Membrane Interactions: Dependence on Membrane Lipid Composition. *Journal of Urology*, *155*(3), 1094–1098. https://doi.org/10.1016/S0022-5347(01)66398-5
- Biglar, M., Salehabadi, H., Jabbari, S., Dabirmanesh, B., Khajeh, K., & Mojab, F. (2021). Screening and identification of herbal urease inhibitors using surface plasmon resonance biosensor. *Research Journal of Pharmacognosy*, *8*(2), 51-62.
- Chalmers, C. (2019). Applied Anatomy and Physiology and the Renal Disease Process. In N. Thomas (Ed.), *Renal Nursing* (1st ed., pp. 21–58). Wiley. https://doi.org/10.1002/9781119413172.ch2
- Chen, C., Lü, J. M., & Yao, Q. (2016). Hyperuricemia-related diseases and xanthine oxidoreductase (XOR) inhibitors: an overview. *Medical science monitor: international medical journal of experimental and clinical research*, *22*, 2501.
- Chen, C.; Li, B.; Huang, R.; Dong, S.; Zhou, Y.; Song, J.; Zeng, X.; Zhang, X. Involvement of Ca2+ and ROS signals in nickel-impaired human sperm function. Ecotoxicol. Environ. Saf. 2022, 231, 113181.
- Cheraft-Bahloul, N., Husson, C., Ourtioualous, M., Sinaeve, S., Atmani, D., Stévigny, C., and Antoine, M. H. (2017). Protective Effects of Pistacia lentiscus L. fruit extract against calcium oxalate monohydrate induced proximal tubular injury. *Journal of ethnopharmacology*, *209*, 248-254.
- Coe, F. L. (2005). Kidney stone disease. *Journal of Clinical Investigation*, *115*(10), 2598–2608. https://doi.org/10.1172/JCI26662
- Courbebaisse, M. (2016). *Lithiase rénale de l'adulte: Des mécanismes au traitement médical préventif*.
- Darabinia, M., Gorji, A. M., & Chabra, A. (2016). Medicinal properties of pomegranate in Quran and Islamic Traditions (Hadith). *International Journal of Humanities and Cultural Studies*, *1*(1), 1591-601.
- Daudon, M., Bader, C. A., Jungers, P., Beaugendre, O., & Hoarau, M. P. (1993). *Urinary Calculi: Review of Classification Methods and Correlations with Etiology*.
- Daudon, M., Dessombz, A., Frochot, V., Letavernier, E., Haymann, J.-P., Jungers, P., & Bazin, D. (2016). Comprehensive morpho-constitutional analysis of urinary stones improves etiological diagnosis and therapeutic strategy of nephrolithiasis. *Comptes Rendus. Chimie*, *19*(11–12), 1470–1491. https://doi.org/10.1016/j.crci.2016.05.008
- Devi, A. T., Nagaraj, R., Prasad, A., Lakkappa, D. B., Zameer, F., & Nagalingaswamy, N. P. M. (2023). Nephrolithiasis: Insights into Biomimics, Pathogenesis, and Pharmacology. *Clinical Complementary Medicine and Pharmacology*, *3*(2), 100077. https://doi.org/10.1016/j.ccmp.2022.100077
- Dong, C., Zhou, J., Su, X., He, Z., Song, Q., Song, C., Ke, H., Wang, C., Liao, W., & Yang, S. (2024). Understanding formation processes of calcareous nephrolithiasis in renal interstitium and tubule lumen. *Journal of Cellular and Molecular Medicine*, *28*(7), e18235. https://doi.org/10.1111/jcmm.18235
- Dröge, W. (2002). Free Radicals in the Physiological Control of Cell Function. *Physiological Reviews*, *82*(1), 47–95. https://doi.org/10.1152/physrev.00018.2001
- Eddebbagh, M., Messaoudi, M., Abourriche, A., Berrada, M., Attaleb, M., Benbacer, L., & Bennamara, A. (2016). Correlation of the cytotoxic and antioxidant activities of moroccan pomegranate (Punica granatum) with phenolic and flavonoid contents. *Journal of Pharmacy and Pharmacology*, *4*(9), 511-519.
- El Habbani, R., Lahrichi, A., Sqalli Houssaini, T., Kachkoul, R., Mohim, M., Chouhani, B. A., & Chaqroune, A. (2021). In vitro mass reduction of calcium oxalate urinary calculi by some medicinal plants. *African Journal of Urology*, *27*, 1-6.
- Erkan, M., & Dogan, A. (2018). Pomegranate/Roma—Punica granatum. In *Exotic Fruits* (pp. 355–361). *Elsevier*. https://doi.org/10.1016/B978-0-12-803138-4.00049-6

- Erkan, M., & Kader, A. A. (2011). Pomegranate (Punica granatum L.). In *Postharvest Biology and Technology of Tropical and Subtropical Fruits* (pp. 287–313e). Elsevier. https://doi.org/10.1533/9780857092618.287
- Espinosa-Ortiz, E. J., Eisner, B. H., Lange, D., & Gerlach, R. (2019). Current insights into the mechanisms and management of infection stones. *Nature Reviews Urology*, *16*(1), 35–53. https://doi.org/10.1038/s41585-018-0120-z
- Fedder, M. D., Jakobsen, H. B., Giversen, I., Christensen, L. P., Parner, E. T., & Fedder, J. (2014). An extract of pomegranate fruit and galangal rhizome increases the numbers of motile sperm: a prospective, randomised, controlled, double-blinded trial. *PloS one*, *9*(10), e108532.
- Fellah, B., Bannour, M., Rocchetti, G., Lucini, L., & Ferchichi, A. (2018). Phenolic profiling and antioxidant capacity in flowers, leaves and peels of Tunisian cultivars of Punica granatum L. *Journal of Food Science and Technology*, *55*(9), 3606–3615. https://doi.org/10.1007/s13197-018-3286-8
- Firuzi, O., Lacanna, A., Petrucci, R., Marrosu, G., & Saso, L. (2005). Evaluation of the antioxidant activity of flavonoids by "ferric reducing antioxidant power" assay and cyclic voltammetry. *Biochimica et Biophysica Acta (BBA)-General Subjects*, *1721*(1-3), 174-184.
- Fleisch, H. (1978). Inhibitors and promoters of stone formation. *Kidney International*, *13*(5), 361–371. https://doi.org/10.1038/ki.1978.54
- Frochot, V., & Daudon, M. (2016). Clinical value of crystalluria and quantitative morphoconstitutional analysis of urinary calculi. *International Journal of Surgery*, *36*, 624–632. https://doi.org/10.1016/j.ijsu.2016.11.023
- Geraghty, RM., Jones, P., Somani, BK. (2017). Worldwide trends of urinary stone disease treatment over the last two decades: a systematic review. *Journal of Endourology* 31(6):547-556. https://doi.org/10.1089/end.2016.0895
- Ghadimi, M., Sharifi, S. D., Najafi, A., & Mohammadi, H. (2024). Gallic acid supplementation partially ameliorates reproductive aging in rooster breeders by improving semen quality, sperm kinetics, hormones, and antioxidant status. *Poultry Science*, *103*(7), 103842.
- Gigliobianco, M. R., Cortese, M., Nannini, S., Di Nicolantonio, L., Peregrina, D. V., Lupidi, G., Vitali, L. A., Bocchietto, E., Di Martino, P., & Censi, R. (2022). Chemical, Antioxidant, and Antimicrobial Properties of the Peel and Male Flower By-Products of Four Varieties of Punica granatum L. Cultivated in the Marche Region for Their Use in Cosmetic Products. *Antioxidants*, *11*(4), 768. https://doi.org/10.3390/antiox11040768
- Gil-Martín, E., Forbes-Hernández, T., Romero, A., Cianciosi, D., Giampieri, F., & Battino, M. (2022). Influence of the extraction method on the recovery of bioactive phenolic compounds from food industry by-products. *Food Chemistry*, *378*, 131918. https://doi.org/10.1016/j.foodchem.2021.131918
- Grases, F., Costa-Bauzá, A., & Garcıa-Ferragut, L. (1998). Biopathological crystallization: a general view about the mechanisms of renal stone formation. *Advances in colloid and interface science*, *74*(1-3), 169-194.
- Guerrero-Solano, J. A., Jaramillo-Morales, O. A., Jiménez-Cabrera, T., Urrutia-Hernández, T. A., Chehue-Romero, A., Olvera-Hernández, E. G., & Bautista, M. (2020). Punica protopunica Balf., the Forgotten Sister of the Common Pomegranate (Punica granatum L.): Features and Medicinal Properties—A Review. *Plants*, *9*(9), 1214. https://doi.org/10.3390/plants9091214
- Hajimahmoodi, M., Moghaddam, G., Ranjbar, A. M., Khazani, H., Sadeghi, N., Oveisi, M. R., & Jannat, B. (2013). Total phenolic, flavonoids, tannin content and antioxidant power of some Iranian pomegranate flower cultivars (Punica granatum L.). *American Journal of Plant Sciences*, *4*(09), 1815.
- Harmon, W.J., Sershon, P.D., Blute, M.L., Patterson, D.E., Segura, J.W. (1997). Ureteroscopy: current practice and long-term complications. *Journal of urology*. 157 (1), 28–32.

- Holland, D., Hatib, K., & Bar-Ya'akov, I. (2009). Pomegranate: Botany, Horticulture, Breeding. In J. Janick (Ed.), *Horticultural Reviews* (1st ed., pp. 127–191). Wiley. https://doi.org/10.1002/9780470593776.ch2
- Hook, K. A., & Fisher, H. S. (2020). Methodological considerations for examining the relationship between sperm morphology and motility. *Molecular reproduction and development*, *87*(6), 633-649.
- Hyams, E.S., Munver, R., Bird, V.G., Uberoi, J., Shah, O., 2010. Flexible ureterorenoscopy and holmium laser lithotripsy for the management of renal stone burdens that measure 2 to 3 cm: a multi-institutional experience. *Journal of endourology*. 24 (10), 1583–1588.
- Ismail, H. T. H. (2022). Toxic effects of excess exposure to boric acid on serum biochemical aspect, hematology and histological alterations and ameliorative potential role of melatonin in rats. *Saudi Journal of Biological Sciences*, *29*(10), 103425.
- Jiménez-Zamora, A., Delgado-Andrade, C., & Rufián-Henares, J. A. (2016). Antioxidant capacity, total phenols and color profile during the storage of selected plants used for infusion. *Food Chemistry*, *199*, 339–346. https://doi.org/10.1016/j.foodchem.2015.12.019
- Jouad, H., Haloui M., Rhiouani H., El Hilaly J., Eddouks M. (2001). Ethnobotanical survey of medicinal plants used for the treatment of diabetes, cardiac and renal diseases in the North centre region of Morocco (Fez–Boulemane), *Journal of Ethnopharmacology*. 77175–182. https://doi.org/10.1016/S0378-8741(01) 00289-6
- Jung, H. D., Cho, S., & Lee, J. Y. (2023). Update on the Effect of the Urinary Microbiome on Urolithiasis. *Diagnostics*, *13*(5), 951. https://doi.org/10.3390/diagnostics13050951
- Kachkoul, R., Benjelloun Touimi, G., El Mouhri, G., El Habbani, R., & Lahrichi, A. (2023). Pathophysiological aspects of renal stone formation and stone types. *Notulae Scientia Biologicae*, *15*(1), 11462. https://doi.org/10.55779/nsb15111462
- Kamata, H., & Hirata, H. (1999). Redox Regulation of Cellular Signalling. *Cellular Signalling*, *11*(1), 1–14. https://doi.org/10.1016/S0898-6568(98)00037-0
- Kanwal, Khan, M., Arshia, Khan, K. M., Parveen, S., Shaikh, M., Fatima, N., & Choudhary, M. I. (2019). Syntheses, in vitro urease inhibitory activities of urea and thiourea derivatives of tryptamine, their molecular docking and cytotoxic studies. *Bioorganic Chemistry*, *83*, 595–610. https://doi.org/10.1016/j.bioorg.2018.10.070
- Karimi, M., Sadeghi, R., & Kokini, J. (2017). Pomegranate as a promising opportunity in medicine and nanotechnology. *Trends in food science & technology*, *69*, 59-73.
- Karthikeyan, G., & Vidya, A. K. (2019). Phytochemical analysis, antioxidant and antibacterial activity of pomegranate peel. *Res. J. Life Sci. Bioinform. Pharm. Chem. Sci*, *5*(1), 218.
- Ken Okamoto , Bryan T. Eger , Tomoko Nishino , Emil F. Pai & Takeshi Nishino (2008) Mechanism of Inhibition of Xanthine Oxidoreductase by Allopurinol: Crystal Structure of Reduced Bovine Milk Xanthine Oxidoreductase Bound with Oxipurinol, *Nucleosides, Nucleotides, and Nucleic* Acids, 27:6-7, 888-893, DOI: 10.1080/15257770802146577
- Khan, R. (2011). Antioxidants and poultry semen quality. World's Poult. Sci. J. 67:297–308.
- Khan, S. R. (1997). Calcium Phosphate/Calcium Oxalate Crystal Association in Urinary Stones: Implications for Heterogeneous Nucleation of Calcium Oxalate. *Journal of Urology*, *157*(1), 376–383. https://doi.org/10.1016/S0022-5347(01)65381-3
- Khan, S. R. (2014). Reactive oxygen species, inflammation and calcium oxalate nephrolithiasis. *Translational Andrology and Urology*, *3*(3).
- Khan, S. R., & Canales, B. K. (2023). Proposal for pathogenesis-based treatment options to reduce calcium oxalate stone recurrence. *Asian Journal of Urology*, *10*(3), 246–257. https://doi.org/10.1016/j.ajur.2023.01.008
- Khan, S. R., & Hackett, R. L. (1993). Role of Organic Matrix in Urinary Stone Formation: An Ltrastructural Study of Crystal Matrix Interface of Calcium Oxalate Monohydrate Stones. *Journal of Urology*, *150*(1), 239–245. https://doi.org/10.1016/S0022-5347(17)35454-X

- Khan, S. R., Canales, B. K., & Dominguez-Gutierrez, P. R. (2021). Randall's plaque and calcium oxalate stone formation: Role for immunity and inflammation. *Nature Reviews Nephrology*, *17*(6), 417–433. https://doi.org/10.1038/s41581-020-00392-1
- Khan, S. R., Pearle, M. S., Robertson, W. G., Gambaro, G., Canales, B. K., Doizi, S., Traxer, O., & Tiselius, H.-G. (2016). Kidney stones. *Nature Reviews Disease Primers*, *2*(1), 16008. https://doi.org/10.1038/nrdp.2016.8
- Krajewska, B., & Brindell, M. (2016). Thermodynamic study of competitive inhibitors' binding to urease. *Journal of Thermal Analysis and Calorimetry*, *123*, 2427-2439.
- Kumari, I., Kaurav, H., & Chaudhary, G. (2021). Punica granatum L. (Dadim) Punica granatum L. (Dadim), Therapeutic Importance of World's Most Ancient Fruit Plant. *Journal of Drug Delivery and Therapeutics*, *11*(3), 113–121. https://doi.org/10.22270/jddt.v11i3.4832
- Li, Y., Guo, C., Yang, J., Wei, J., Xu, J., & Cheng, S. (2006). Evaluation of antioxidant properties of pomegranate peel extract in comparison with pomegranate pulp extract. *Food Chemistry*, *96*(2), 254–260. https://doi.org/10.1016/j.foodchem.2005.02.033
- Li, Z., Wang, H., Sun, S., Shao, Z., Lv, C., Dong, X., ... & Wang, W. (2024). Ellagitannins from pomegranate (Punica granatum L.) flower with xanthine oxidase and α-glucosidase inhibitory activities. *Journal of Functional Foods*, *116*, 106153.
- Liu, F., Deng, C., Cao, W., Zeng, G., Deng, X., & Zhou, Y. (2017). Phytochemicals of Pogostemon Cablin (Blanco) Benth. aqueous extract: Their xanthine oxidase inhibitory activities. Biomedicine & Pharmacotherapy, 89, 544–548. https://doi.org/10.1016/j.biopha.2017.01.040
- Liu, L., Zhang, L., Ren, L., & Xie, Y. (2020). Advances in structures required of polyphenols for xanthine oxidase inhibition. *Food Frontiers*, *1*(2), 152-167.
- Madrigal-Carballo S., Rodriguez G., Krueger CG., Dreher M., Reed JD. (2009) Pomegranate (Punica granatum) supplements: Authenticity, antioxidant and polyphenol composition. J Funct Foods 1: 324–9.
- Maphetu, N., Unuofin, J. O., Masuku, N. P., Olisah, C., & Lebelo, S. L. (2022). Medicinal uses, pharmacological activities, phytochemistry, and the molecular mechanisms of Punica granatum L. (pomegranate) plant extracts: A review. *Biomedicine & Pharmacotherapy*, *153*, 113256. https://doi.org/10.1016/j.biopha.2022.113256
- Martinez-Pastor, F., Garcia-Macias, V., Alvarez, M., Chamorro, C., Herraez, P., de Paz, P., & Anel, L. (2006). Comparison of two methods for obtaining spermatozoa from the cauda epididymis of Iberian red deer. *Theriogenology*, *65*(3), 471-485.
- Materska, M. (2015). Flavone C-glycosides from Capsicum annuum L.: Relationships between antioxidant activity and lipophilicity. European Food Research and Technology, 240(3), 549–557. https://doi.org/10.1007/ s00217-014-2353-2
- McInnes, G. T., Lawson, D. H., & Jick, H. (1981). Acute adverse reactions attributed to allopurinol in hospitalised patients. *Annals of the Rheumatic Diseases*, *40*(3), 245-249.
- Meng, X.-Y., Zhang, H.-X., Mezei, M., & Cui, M. (2011). Molecular Docking: A Powerful Approach for Structure-Based Drug Discovery. *Current Computer Aided-Drug Design*, *7*(2), 146–157. https://doi.org/10.2174/157340911795677602
- Miller, N. L., Evan, A. P., & Lingeman, J. E. (2007). Pathogenesis of Renal Calculi. *Urologic Clinics of North America*, *34*(3), 295–313. https://doi.org/10.1016/j.ucl.2007.05.007
- Moe, O. W. (2006). *Kidney stones: Pathophysiology and medical management*. *367*.
- Moretti, E., Signorini, C., Corsaro, R., Giamalidi, M., & Collodel, G. (2023). Human sperm as an in vitro model to assess the efficacy of antioxidant supplements during sperm handling: A narrative review. *Antioxidants*, *12*(5), 1098.
- Morris, GM., Huey, R., Lindstrom, W., Sanner, MF., Belew, RK., Goodsell, DS., Olson, AJ, (2009). AutoDock4 and AutoDockTools4: Automated docking with selective receptor flexibility. J Comput Chem, 30, (16), 2785-91. https://doi.org/10.7324/JABB.2020.80117

- Moussa, M., Papatsoris, A. G., Abou Chakra, M., & Moussa, Y. (2020). Update on cystine stones: Current and future concepts in treatment. *Intractable & Rare Diseases Research*, *9*(2), 71–78. https://doi.org/10.5582/irdr.2020.03006
- Munteanu, I. G., & Apetrei, C. (2021). Analytical Methods Used in Determining Antioxidant Activity: A Review. *International Journal of Molecular Sciences*, *22*(7), 3380. https://doi.org/10.3390/ijms22073380
- Nabati, F., Mojab, F., Habibi-Rezaei, M., Bagherzadeh, K., Amanlou, M., & Yousefi, B. (2012). Large scale screening of commonly used Iranian traditional medicinal plants against urease activity. *DARU Journal of Pharmaceutical Sciences*, *20*, 1-9.
- Nagao, M. Seki., and Kobayashi, H., (1999). "Inhibition of xanthine oxidase by flavonoids," Biosci Biotechnol Bioche. 63,10, 1787-1790.
- Nile, S. H., Nile, A. S., Keum, Y. S., & Sharma, K. (2017). Utilization of quercetin and quercetin glycosides from onion (Allium cepa L.) solid waste as an antioxidant, urease and xanthine oxidase inhibitors. Food Chemistry, 235, 119–126. https://doi.org/10.1016/j.foodchem.2017.05.043
- Nirumand, M., Hajialyani, M., Rahimi, R., Farzaei, M., Zingue, S., Nabavi, S., & Bishayee, A. (2018). Dietary Plants for the Prevention and Management of Kidney Stones: Preclinical and Clinical Evidence and Molecular Mechanisms. *International Journal of Molecular Sciences*, *19*(3), 765. https://doi.org/10.3390/ijms19030765
- Owen, P. L., & Johns, T. (1999). Xanthine oxidase inhibitory activity of northeastern North American plant remedies used for gout. *Journal of Ethnopharmacology*, *64*(2), 149-160. https://doi.org/10.1016/S0378-8741(98)00119-6
- Paliouras, C., Tsampikaki, E., Alivanis, P., & Aperis, G. (2012). Pathophysiology of Nephrolithiasis. *Nephrology Research & Reviews*, *4*(2), 58–65. https://doi.org/10.4081/nr.2012.e14
- Panth, N., Manandhar, B., & Paudel, K. R. (2017). Anticancer Activity of *Punica granatum* (Pomegranate): A Review: Anticancer Activity of Pomegranate. *Phytotherapy Research*, *31*(4), 568–578. https://doi.org/10.1002/ptr.5784
- Patil R., Das S., Stanley A., Yadav L., Sudhakar A., Varma AK. (2010). Optimized hydrophobic interactions and hydrogen bonding at the target-ligand interface leads the pathways of drug-designing. PLoS One. 16;5(8): e12029. doi: 10.1371/journal.pone.0012029
- Peerapen, P., & Thongboonkerd, V. (2023). Kidney Stone Prevention. *Advances in Nutrition*, *14*(3), 555–569. https://doi.org/10.1016/j.advnut.2023.03.002
- Peršurić, Ž., Saftić Martinović, L., Malenica, M., Gobin, I., Pedisić, S., Dragović-Uzelac, V., & Kraljević Pavelić, S. (2020). Assessment of the Biological Activity and Phenolic Composition of Ethanol Extracts of Pomegranate (Punica granatum L.) Peels. *Molecules*, *25*(24), 5916. https://doi.org/10.3390/molecules25245916
- Petrova, I., Petkova, N., Ivanov, I., Todorova, M., Ognyanov, M., Bileva, T., & Haytova, D. (n.d.). *Bioactive Compounds And Antioxidant Activity Of Extracts From Edible Flowers Of Punica Granatum And Citrus Aurantium*.
- Pulido, R., Bravo, L., & Saura-Calixto, F. (2000). Antioxidant Activity of Dietary Polyphenols As Determined by a Modified Ferric Reducing/Antioxidant Power Assay. *Journal of Agricultural and Food Chemistry*, *48*(8), 3396–3402. https://doi.org/10.1021/jf9913458
- Qing-qing Han, Qi-dong Ren, Xu Guo, Mohamed A. Farag, Yu-hong Zhang, Meng-qi Zhang, Ying-ying Chen, Shu-tao Sun, Jin-yue Sun, Ning-yang Li, Chao Liu. (2024). Punicalagin attenuates hyperuricemia via restoring hyperuricemia-induced renal and intestinal dysfunctions, Journal of Advanced Research. https://doi.org/10.1016/j.jare.2024.03.029
- Ramírez, D., Caballero, J. (2018). Is It Reliable to Take the Molecular Docking Top Scoring Position as the Best Solution without Considering Available Structural Data? Molecules, 23, 1038. https://doi.org/10.3390/molecules23051038)

- Romero, V., Akpinar, H., & Assimos, D. G. (2010). *Kidney Stones: A Global Picture of Prevalence, Incidence, and Associated Risk Factors*.
- Rozadi N., Oktavia S., Fauziah F. Pharmacological Activities of Punicalagin: A Review. Journal of Drug Delivery and Therapeutics, 12(2), 148-155.
- Rummun, N., Somanah, J., Ramsaha, S., Bahorun, T., & Neergheen-Bhujun, V. S. (2013). Bioactivity of Nonedible Parts of *Punica granatum* L.: A Potential Source of Functional Ingredients. *International Journal of Food Science*, *2013*, 1–12. https://doi.org/10.1155/2013/602312
- Saeed, A., Mahesar, P. A., Channar, P. A., Larik, F. A., Abbas, Q., Hassan, M., Raza, H., & Seo, S.-Y. (2017). Hybrid pharmacophoric approach in the design and synthesis of coumarin linked pyrazolinyl as urease inhibitors, kinetic mechanism and molecular docking. Chemistry & Biodiversity, 14, e1700035.
- Sakhaee, K. (2008). Nephrolithiasis as a systemic disorder: *Current Opinion in Nephrology and Hypertension*, *17*(3), 304–309. https://doi.org/10.1097/MNH.0b013e3282f8b34d
- Setti, A. S., Braga, D. P. D. A. F., Provenza, R. R., Iaconelli Jr, A., & Borges Jr, E. (2021). Oocyte ability to repair sperm DNA fragmentation: the impact of maternal age on intracytoplasmic sperm injection outcomes. *Fertility and Sterility*, *116*(1), 123-129.
- Shah, J., & Whitfield, H. N. (2002). Urolithiasis through the ages. *BJU International*, *89*(8), 801–810. https://doi.org/10.1046/j.1464-410X.2002.02769.x
- Shaliutina, O., Materiienko, A., Shaliutina-Kolešová, A., & Gazo, I. (2021). Using fish spermatozoa in in vitro toxicity tests: A potential toxicology tool. *Aquaculture*, *539*, 736647.
- Sharifi-Rad J, Quispe C, Castillo CMS, Caroca R, Lazo-Vélez MA, Antonyak H, Polishchuk A, Lysiuk R, Oliinyk P, De Masi L, Bontempo P, Martorell M, Daştan SD, Rigano D, Wink M, Cho WC. (2022). Ellagic Acid: A Review on Its Natural Sources, Chemical Stability, and Therapeutic Potential. Oxid Med Cell Longev 2022, 1, 3848084. doi: 10.1155/2022/3848084.
- Shckorbatov, Y. G., Shakhbazov, V. G., Bogoslavsky, A. M., & Rudenko, A. O. (1995). On age-related changes of cell membrane permeability in human buccal epithelium cells. *Mechanisms of Ageing and Development*, *83*(2), 87–90. https://doi.org/10.1016/0047-6374(95)93574-M
- Sikarwar, I., Dey, YN., Wanjari, MM., Sharma, A., Gaidhani, SN., Jadhav AD. (2017). Chenopodium album Linn. leaves prevent ethylene glycol-induced urolithiasis in rats. J*ournal of Ethnopharmacology* 195:275-282. https://doi.org/10.1016/j.jep.2016.11.031
- Singh, P., Enders, F. T., Vaughan, L. E., Bergstralh, E. J., Knoedler, J. J., Krambeck, A. E., Lieske, J. C., & Rule, A. D. (2015). Stone Composition Among First-Time Symptomatic Kidney Stone Formers in the Community. *Mayo Clinic Proceedings*, *90*(10), 1356–1365. https://doi.org/10.1016/j.mayocp.2015.07.016
- Singh, S. R., & Hou, S. X. (2009). Multipotent stem cells in the Malpighian tubules of adult Drosophila melanogaster. *Journal of Experimental Biology*, *212*(3), 413-423.
- Sohgaura, A., & Bigoniya, P. (2017). A Review on Epidemiology and Etiology of Renal Stone. *American Journal of Drug Discovery and Development*, *7*(2), 54–62. https://doi.org/10.3923/ajdd.2017.54.62
- Stamatelou, K. K., Francis, M. E., Jones, C. A., Nyberg, L. M., & Curhan, G. C. (2003). Time trends in reported prevalence of kidney stones in the United States: 1976–199411.See Editorial by Goldfarb, p. 1951. *Kidney International*, *63*(5), 1817–1823. https://doi.org/10.1046/j.1523-1755.2003.00917.x
- Stamatelou, K., & Goldfarb, D. S. (2023). Epidemiology of Kidney Stones. *Healthcare*, *11*(3), 424. https://doi.org/10.3390/healthcare11030424
- Sweidan, N., Abu Rayyan, W., Mahmoud, I., & Ali, L. (2023). Phytochemical analysis, antioxidant, and antimicrobial activities of Jordanian Pomegranate peels. *PLOS ONE*, *18*(11), e0295129. https://doi.org/10.1371/journal.pone.0295129

- Talso, M., Tefik, T., Mantica, G., Rodriguez Socarras, M., Kartalas Goumas, I., Somani, B.,.K., Esperto, F., (2019). Extracorporeal shockwave lithotripsy: current knowledge and future perspectives. Minerva Urol. Nephrol. 71 (4), 365–372.
- Tamborino, F., Cicchetti, R., Mascitti, M., Litterio, G., Orsini, A., Ferretti, S., Basconi, M., De Palma, A., Ferro, M., Marchioni, M., & Schips, L. (2024). Pathophysiology and Main Molecular Mechanisms of Urinary Stone Formation and Recurrence. *International Journal of Molecular Sciences*, *25*(5), 3075. https://doi.org/10.3390/ijms25053075
- Tan, Z., Zhou, J., Chen, H., Zou, Q., Weng, S., Luo, T., & Tang, Y. (2016). Toxic effects of 2, 4-dichlorophenoxyacetic acid on human sperm function in vitro. *The Journal of Toxicological Sciences*, *41*(4), 543-549.
- Tandon, C., Chaudhary, A., & Singla, S. (2010). In vitro evaluation of Terminalia arjuna on calcium phosphate and calcium oxalate crystallization. *Indian Journal of Pharmaceutical Sciences*, *72*(3), 340. https://doi.org/10.4103/0250-474X.70480
- Tavasoli, S., & Taheri, M. (2019). Vitamin D and calcium kidney stones: A review and a proposal. *International Urology and Nephrology*, *51*(1), 101–111. https://doi.org/10.1007/s11255-018-1965-z
- Taylor, J. (2023). Renal system 1: the anatomy and physiology of the kidneys. *Nursing Times*, 119 Issue 21
- The Consensus Conference Group, Gambaro, G., Croppi, E., Coe, F., Lingeman, J., Moe, O., Worcester, E., Buchholz, N., Bushinsky, D., Curhan, G. C., Ferraro, P. M., Fuster, D., Goldfarb, D. S., Heilberg, I. P., Hess, B., Lieske, J., Marangella, M., Milliner, D., Preminger, G. M., … Williams, J. C. (2016). Metabolic diagnosis and medical prevention of calcium nephrolithiasis and its systemic manifestations: A consensus statement. *Journal of Nephrology*, *29*(6), 715–734. https://doi.org/10.1007/s40620-016-0329-y
- Torricelli, F.C.M., Danilovic, A., Vicentini, F.C., Marchini, G.S., Srougi, M., Mazzucchi, E. (2015). Extracorporeal shock wave lithotripsy in the treatment of renal and ureteral stones. *Revista da associaçao medica Brasileira*. 6 (1), 65–71
- Tran, S. K., Huynh, B. T., Vo, C. T., Van Ngo, T., Tran, B. L. T., Tran, K. D. D., ... & Tran, A. V. (2024). Treatment efficacy of febuxostat compared with allopurinol in hyperuricemia patients with hypertensive: A randomized, single-blind controlled trial. *Journal of Applied Pharmaceutical Science*, *14*(6), 090-096.
- Tremellen, K. (2008) Oxidative Stress and male infertility – a clinical perspective. Hum Reprod Update 14: 243–258.
- Turk G, Sonmez M, Aydin M, Yuce A, Gur S, et al. (2008) Effects of pomegranate juice consumption on sperm quality, spermatogenic cell density, antioxidant activity and testosterone level in male rats. Clin Nutr, 27: 289–296.
- Umamaheswari, M., Madeswaran, A., Asokkumar, K., Sivashanmugam, T., Subhadradevi, V., & Jagannath, P. (2011). Study of potential xanthine oxidase inhibitors: In silico and in vitro biological activity. Bangladesh Journal of Pharmacology, 6(2), 117-123.)
- Venkatesan, N., Shroff, S., Jeyachandran, K., & Doble, M. (2011). Effect of uropathogens on in vitro encrustation of polyurethane double J ureteral stents. *Urological Research*, *39*(1), 29–37. https://doi.org/10.1007/s00240-010-0280-7
- Vijeesh, V., Jisha, N., Vysakh, A., & Latha, M.S. (2021). Interaction of eugenol with xanthine oxidase: Multi spectroscopic and in silico modelling approach. Spectrochimica Acta Part A: Molecular and Biomolecular Spectroscopy, 258, 119843. https://doi.org/10.1016/j.saa.2021.119843
- Vollmer, T., Ljungberg, B., Jankowski, V., Jankowski, J., Glorieux, G., & Stegmayr, B. G. (2019). An in-vitro assay using human spermatozoa to detect toxicity of biologically active substances. *Scientific Reports*, *9*(1), 14525.
- Wallace (1998). Anatomv and physiology of the kidney. Aorn journal, 68, no 5

- Weatherburn, M. (1967). Phenol-hypochlorite reaction for determination of ammonia. *Analytical chemistry*, *39*(8), 971-974.
- Wigner, P., Grębowski, R., Bijak, M., Szemraj, J., & Saluk-Bijak, J. (2021). The Molecular Aspect of Nephrolithiasis Development. *Cells*, *10*(8), 1926. https://doi.org/10.3390/cells10081926
- Williams-Larson, A. W. (1990). Urinary calculi associated with purine metabolism: uric acid nephrolithiasis. *Endocrinology and metabolism clinics of North America*, *19*(4), 821-838.
- Wong, Y. P., Ng, R. C., Chuah, S. P., Koh, R. Y., & Ling, A. P. K. (2014, August). Antioxidant and xanthine oxidase inhibitory activities of Swietenia macrophylla and Punica granatum. In *International Conference on Biological, Environment and Food Engineering (BEFE-2014), August* (pp. 4-5).
- **World Health Organization,** Cooper, T. G., Noonan, E., Von Eckardstein, S., Auger, J., Baker, H. G., Behre, H. M., ... & Vogelsong, K. M. (2010). World Health Organization reference values for human semen characteristics. *Human reproduction update*, *16*(3), 231-245.
- Xu, B., Wang, Z. P., Wang, Y. J., Lu, P. H., Wang, L. J., & Wang, X. H. (2013). The toxic effect of opioid analgesics on human sperm motility in vitro. *Drug and Chemical Toxicology*, *36*(2), 205-208.
- Yuan, M., Liu, Y., Xiao, A., Leng, J., Liao, L., Ma, L., & Liu, L. (2019). The interaction of dietary flavonoids with xanthine oxidase in vitro: Molecular property-binding affinity relationship aspects. RSC Advances, 9(19), 10781–10788. https://doi.org/10.1039/C8RA09926J
- Zeghad, N., Abassi, E. A., Belkhiri, A., Demeyer, K., & Heyden, Y. V. (2022). Phenolic compounds profile from Algerian pomegranate fruit extract (Punica granatum L.) by UPLC-DAD-ESI-MS. *Chemistry Africa*, *5*(5), 1295-1303.
- Zhang, C., Zhang, G., Pan, J., & Gong, D. (2016). Galangin competitively inhibits xanthine oxidase by a ping-pong mechanism. Food Research International, 89, 152–160. https://doi.org/10.1016/j.foodres.2016.07.021.
- Zhang, J. Q., Jiang, K. M., An, K., Ren, S. H., Xie, X. G., Jin, Y., & Lin, J. (2015). Novel water-soluble fisetin/cyclodextrins inclusion complexes: Preparation, characterization, molecular docking and bioavailability. *Carbohydrate research*, *418*, 20-28.
- Zhang, L., Wang, X., Sohail, T., Jiang, C., Sun, Y., Wang, J., and Li, Y. (2024). Punicalagin Protects Ram Sperm from Oxidative Stress by Enhancing Antioxidant Capacity and Mitochondrial Potential during Liquid Storage at 4° C. *Animals*, *14*(2), 318.
- Zhizhou Li, Hui Wang, Shiwei Sun, Zhongbai Shao, Chaoyi Lv, Xiaoyue Dong, Lu Wang, Wei Wang. (2024). Ellagitannins from pomegranate (Punica granatum L.) flower with xanthine oxidase and α-glucosidase inhibitory activities. Journal of Functional Foods, 116, 106153, https://doi.org/10.1016/j.jff.2024.106153

Appendices

Appendix I

Table I: Recommended medication based on the specific types of stones as suggested by. European Association of Urology 2023 **(Tamborino et *al*., 2024)**

Type of Stones	Suggested Medication
Calcium Oxalate stones	Fluid intake, diet - Hyperoxaluria → foods with low oxalate content and benefits with Calcium (1000 to 2000 mg/d depending on oxalate excretion) and magnesium - Hypercalciuria 5–8 mmol/d → Alkaline citrate or sodium bicarbonate - Hypercalciuria > 8 mmol/d → Hydrochloro-thiazide initially 25 mg/d up to 50 mg/d chlorthalidone 25 mg/d indapamide 2.5 mg/d
Calcium Phosphate stones	Primary hyperparathyroidism → surgery Renal tubular acidosis → bicarbonate or alkaline citrate therapy Fluid intake, diet Hyperuricosuria → purine reduction in their daily diet. Alkaline citrate or sodium bicarbonate plus/or allopurinol
Uric acid stones	- Hyperuricosuria > 4.0 mmol/d → Allopurinol 100 mg/d - Hyperuricosuria > 4.0 mmol/d or hyperuricaemia > 380 µmol → Allopurinol 100-300 mg/d
Struvite and infection stones	Fluid intake, diet, complete surgical stone removal, short- or long-term antibiotic treatment, urinary acidification using methionine or ammonium chloride, and advice to restrict intake of urease
Cystine stones	A 24 h urine volume of >3 L, a diet low in methionine, Avoidance of sodium consumption > 2 g/day, Tiopronin, Captopril

Appendix II

Table II: Definition of spermatozoa kinematics parameters (**Hook & Fisher, 2020).**

Parameter	Acronym	Definition
Straight line velocity	VSL	Calculated using the straight line distance between the first and last detected positions of the sperm head divided by the total elapsed time
Curvilinear velocity	VCL	Calculated by summing the actual curvilinear distance of the sperm cell head and dividing it by the total elapsed time
Average path velocity	VAP	The time-averaged velocity of the sperm head along its average path, which is computed by an algorithm that smooths the curvilinear path

Appendix III

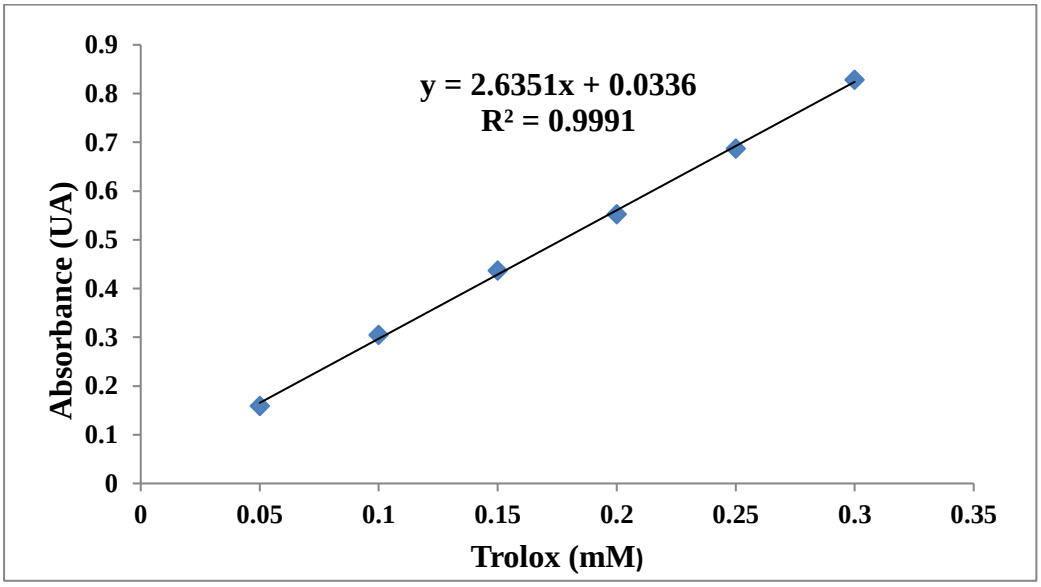

Figure 01: Ferric Reducing Antioxidant Power (FRAP) assay calibration curve using TROLOX as a reference molecule

Abstract

Kidney stones, or urolithiasis, are one of the oldest diseases. Their high incidence and recurrence rate cause a major public health issue, especially with the treatment limitations, as these methods are costly and present severe side effects. That is why current research is expanding to find new alternatives. In ancient medicine, plants were used to heal different diseases. *Punica granatum*, is a common medicinal plant usually used in treating kidney diseases. This work aimed to evaluate the antiurolithiasic activity of the aqueous flower extract (PGF) and ethanolic peel delipidated (PGPd) and non-delipidated (PGPnd) extract on different study models. The *in vitro* models investigated the ferric reducing antioxidant power where both aqueous flower and the ethanolic non delipidated peel extracts gave best activity, while for the enzyme inhibitory test the aqueous flower extract was more efficient against xanthine oxidase (IC_{50}38.11±7.835 µg/mL) while the ethanolic non delipidated peel extract gave best result for inhibiting urease (IC_{50} of 32.61±0.625 µg/mL). These inhibitory activities were further investigated using an *in silico* model determining the interaction of two majority components namely punicalagin and ellagic acid of the *Punica granatum* extracts. Litholytic test on the COM crystals reveled an efficacity of all different extracts compared to the standard. Additionally the investigation was carried using spermatozoa as a semi *in vivo* model, the aqueous flower extract proved great protection activity against COM crystals, confirmed by the amelioration of the motility parameters (VSL,VCL,VAP). Our work proves the validation of spermatozoa, as a future toxicity monitor model for the urolithiasis studies.
Key words: Xanthine oxidase, urease, docking, spermatozoa, COM, *Punica granatum*.

Resumé

Les calculs rénaux, ou l'urolithiase, est l'une des maladies les plus anciennes. Présentant des taux d'incidence et de récidivité élevée devenant un problème majeur de la santé publique, suite aux limitations des traitements, ces méthodes sont coûteuses et présentent des effets secondaires graves. C'est pourquoi la recherche actuelle s'étend pour trouver de nouvelles alternatives. Dans la médecine ancienne, les plantes étaient utilisées pour guérir diverses maladies. *Punica granatum*, est une plante médicinale courante traditionnellement utilisée dans le traitement des maladies rénales. Ce travail visait à évaluer l'activité antiurolithiasique de l'extrait aqueux de fleur (PGF) et l'extrait éthanolique délipidée (PGPd) et non-delipidé (PGPsnd) de l'écorce sur différents modèles d'étude. Les modèles *in vitro* ont étudié le pouvoir antioxydante où l'extrait aqueux de fleurs ainsi que l'extrait éthanolique non délipidés de l'écorce ont donnés la meilleure activité, tandis que pour les tests d'inhibition enzymatique, l'extrait aqueux de fleur était plus efficace contre la xanthine oxidase (IC5038.11±7.835 µg/mL) alors que l'extrait d'éthanolique non délibidée de l'écores a présenté une meilleure activité contre l'uréase (IC 50 de 32.61±0.625 µg / mL). Ces activités inhibitrices ont été investiguées d'avenage en utilisant un modèle *in silico* déterminant l'interaction de deux composants majeurs, à savoir le punicalagine et l'acide ellagique des extraits de *P. granatum*. Le test litholytique sur les cristaux OCM a révélé une efficacité de tous les différents extraits par rapport au standard. En outre, l'étude a été menée en utilisant des spermatozoïdes en tant que modèle *semi in vivo*. L'extrait de aqueux de fleur a démontré une activité protectrice hautement significative contre les cristaux OCM, qui est confirmée par l'amélioration des paramètres de motilité (VSL,VCL,VAP). Notre travail prouve la validation des spermatozoïdes, en tant que futur modèle d'étude de la toxicité pour les études de l'urolithiase.
Mots clés : Xanthine oxidase, urease, docking, spermatozoïde, OCM, *Punica granatum*

ملخص

حصى الكلى، أو تحص بولي، هي واحدة من أقدم الامراض، يتسبب معدل حدوثها وتكرارها المرتفع في مشكلة صحية عامة كبيرة، خاصة مع قيود العلاج، لأن هذه الطرق مكلفة وتسبب آثارا جانبية شديدة. هذا هو السبب في أن الأبحاث الحالية تتوسع لإيجاد بدائل جديدة. في الطب القديم، تم استخدام النباتات لعلاج الامراض المختلفة. الرمان، هو نبات طبي شائع يستخدم عادة في علاج أمراض الكلى. يهدف هذا العمل إلى تقييم النشاط المضاد لحصى الكلى لمستخلص مائي لزهرة الرمان ومستخلص قشر الإيثانول منزوع الدهون وغير منزوع الدهون على نماذج دراسة مختلفة .قامت النماذج في المختبر بالتحقيق في قوة مضادات الأكسدة المختزلة للحديد حيث أعطى كل من المستخلص المائي لزهرة الرمان ومستخلصات الإيثانولية للقشور غير منزوعة الدهون أفضل نشاط ، بينما بالنسبة لاختبار مثبطات الإنزيم ، كان المستخلص المائي لزهرة الرمان أكثر كفاءة ضد انزيم الزانثين أوكسيديز (ت م أ 7.835±5038.11 ميكروغرام / مل) في حين أن مستخلص القشور الإيثانولي غير منزوع الدهون أعطى أفضل نتيجة لتثبيط اليورياز(ت م أ 0.625±61 ميكروغرام / مل) تم التحقيق في هذه الأنشطة المثبطة باستخدام نموذج يحدد تفاعل مكونين من المستخلصات. حمض الإيلاجيك وبونيكالاجين .اما كشف اختبار المضاد لحصى الكلى على بلورات أكسالات الكالسيوم مونوهيدرات أعلن فعالية جميع المستخلصات المختلفة مقارنة بالمعيار. بالإضافة إلى ذلك، تم إجراء التحقيق باستخدام الحيوانات المنوية كنموذج شبه حي أثبت المستخلص المائي لزهرة الرمان نشاطا كبيرا للحماية ضد بلورات أكسالات الكالسيوم مونوهيدرات، وهو ما يؤكده تحسن معلمات الحركة ما يثبت التحقق من صحة الحيوانات المنوية، كنموذج لمراقبة السمية في المستقبل لدراسات تحص بولي.

كلمات مفتاحية: أ كسيداز زانثين, إنزيم اليوريز, الالتحام, الحيوانات المنوية, جراناتوم بونيكا .