

Evaluation of the inhibitory effects of phenolic compounds on calcium oxalate crystallization

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Kidney stone disease, a widespread condition with a history dating back to ancient civilizations, remains a significant global health issue. Among the various types of kidney stones, calcium oxalate (CaOx) stones are predominant, accounting for the majority of urinary tract stones. Alongside conventional treatment methods such as surgery, medication, and lithotripsy, there is growing interest in complementary and alternative therapies, including herbal remedies. This study evaluates the inhibitory effects of phenolic compounds on calcium oxalate crystallization—a process of great importance in biomineralization—and assesses their drug-likeness using *in-silico* approaches. The study aims to predict the potential pharmacokinetic properties of flavonoid compounds in the human body using an *in-silico* ADME (absorption, distribution, metabolism, and excretion) approach. *Helichrysum arenarium* (immortelle/golden everlasting), a plant native to various regions of Turkey, was used as the source of the plant extract. The extract was obtained *via* supercritical CO₂ extraction, and the study elucidates, at the molecular level, how flavonoids (such as quercetin, kaempferol, and naringenin) and phenolic compounds (such as chlorogenic acid and caffeic acid) inhibit CaOx crystal formation.

Keywords: Calcium oxalate (CaOx), kidney stone, *Helichrysum arenarium* extract, flavonoids, phenolic compounds.

INTRODUCTION

Crystallization is the process by which a substance separates from a more disordered structure into more ordered solid particles. It basically consists of three stages: nucleation, crystal growth, and aggregation [1, 2]. Although crystallization is an effective method used in fields such as the pharmaceutical industry and purification, food production, or materials science, there are also situations where its effects can be negative and lead to serious problems. Two of these are industrial crystallization and kidney stone formation, a urinary system disorder [3, 4]. Calcium oxalate, particularly in its monohydrate phase, is the main component of typical kidney stones [5, 6]. Kidney stone formation is one of the most significant clinical problems in the human body. Kidney stone disease reduces a person's healthy life expectancy and places a heavy financial burden on healthcare systems; both of these significantly lower an individual's quality of life [7, 8]. Therefore, calcium oxalate crystallization has become an important topic of interest to researchers in biomineralization. Research indicates that traditional medicine, including herbal remedies, is used by individuals in various cultures and countries to treat kidney stones. *Helichrysum arenarium* possesses antibacterial, antiviral, antifungal, anti-inflammatory, antimicrobial, anti-allergic and antioxidant properties and is used in folk medicine

for the treatment of liver and gallbladder disorders, stomach ache, asthma, cystitis and jaundice, skin infections, respiratory and digestive system disorders, kidney stone treatment and urogenital disorders [9, 10].

Recent studies have shown that SwissADME outcomes are widely used in evaluating the bioavailability, renal distribution, and safety characteristics of naturally occurring phenolic compounds and flavonoids investigated as kidney stone inhibitors. In particular, performing SwissADME analyses in conjunction with *in vitro* crystallization experiments allows for the evaluation of active compounds not only as crystal inhibitors but also as potential therapeutic candidates [11-14].

EXPERIMENTAL

Materials and method

- *Extraction of Helichrysum arenarium using supercritical fluid extraction (SFE) method.* The leaves of the *Helichrysum arenarium* plant were sourced from a local herbalist in Uşak. The *Helichrysum arenarium* extract, used as a potential inhibitor of calcium oxalate monohydrate growth, was obtained using a supercritical fluid extraction method. Food-grade carbon dioxide (CO₂) with 99.9% purity, stored in a bottom tube, was used as the extraction solvent; ethanol with 99.9% purity was used as a co-solvent to increase the solubility of polar compounds in supercritical CO₂.

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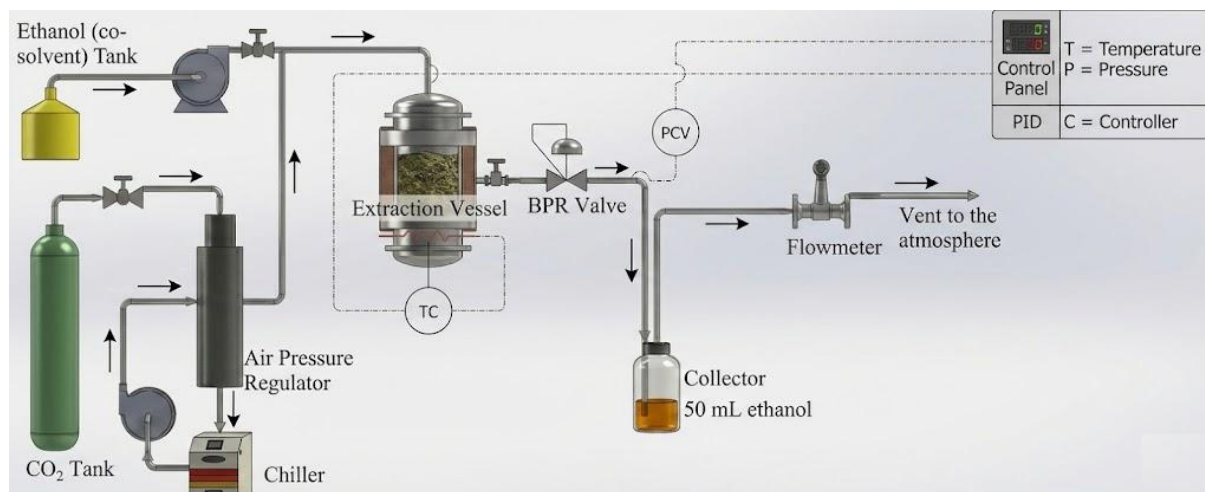


Figure 1. Experimental setup

Supercritical CO₂ extraction (SCF) conditions were as follows: 5 L/min CO₂ flow rate, 15% v/w ethanol ratio, 400 bar extraction pressure and 40 °C temperature. Supercritical fluid extraction experiments on *Achillea millefolium* were conducted using a laboratory-scale supercritical fluid extraction system (Applied Separations, USA), as shown schematically in Figure 1.

- **Phenolic content analysis by LC-HRMS:** The phenolic component profile of *Helichrysum arenarium*, *Thymus vulgaris* L., and *Achillea millefolium* extracts obtained by supercritical CO₂ extraction was determined using the LC-HRMS (liquid chromatography–high resolution mass spectrometry) (Thermo ORBITRAP Q-EXACTIV) method, which combines liquid chromatography with high-resolution mass spectrometry.

- **In-silico evaluation of the inhibitory effects of flavonoids and phenolic compounds:** In evaluating the therapeutic potential of phenolic compounds and flavonoids that exhibit efficacy in preventing kidney stone formation, their *in vitro* crystallization inhibitory activity alone is insufficient. Candidate molecules are expected to demonstrate adequate absorption after oral administration, enter the circulation, reach kidney tissue, and possess acceptable pharmacokinetic properties. Therefore, the SwissADME platform has been widely used in natural product research in recent years.

- **Gastrointestinal (GI) absorption:** Since most natural compounds used in kidney stone treatment are planned to be administered orally, high GI absorption is one of the most important parameters. High gastrointestinal absorption allows the molecule to pass into the systemic circulation at a higher rate, increasing the effective concentration

reaching the kidney. In the literature, gastrointestinal absorption has been the first characteristic evaluated in SwissADME analyses of quercetin, kaempferol, hesperidin, rutin, and various phenolic acids.

- **Bioavailability score:** The bioavailability score indicates the likelihood of bioavailability after oral administration. For phenolic compounds, value of 0.55 is generally considered suitable. It is reported that flavonoids with low bioavailability may have limited *in vivo* efficacy despite being effective *in vitro*.

- **Lipinski rule of five:** Kidney stone inhibitor candidates should be evaluated for compliance with the Lipinski rules in terms of exhibiting drug-like properties. Parameters to be evaluated: molecular weight (<500 Da), H-bond donor, H-bond acceptor, LogP (<5). Lipinski violation can occur in most flavonoid glycosides due to increased molecular weight. In contrast, aglycone forms (e.g., quercetin and kaempferol) generally exhibit more favorable pharmacokinetic properties.

- **Consensus logP:** The logP value indicates lipophilicity. Moderate lipophilicity is preferred for kidney stone inhibitors. Very low logP may make it difficult to cross the cell membrane. Very high logP may reduce solubility. Generally, logP values in the range of 1–3 are considered advantageous in terms of reaching kidney tissue.

- **Water solubility:** For phenolic compounds to be effective in the urinary environment, it is important that they have sufficient water solubility. High solubility facilitates transport in urine, reaching the renal tubules, and interacting with crystals.

- **Topological polar surface area (TPSA):** The TPSA value is an important molecular descriptor associated with membrane permeability and oral absorption. Generally, a TPSA value in the range of

20–140 Å² is considered favorable for oral bioavailability, although this depends on other molecular properties. A high TPSA may reduce passive membrane permeability and intestinal absorption because highly polar molecules have limited ability to partition into the lipophilic environment of biological membranes. Flavonoid glycosides generally exhibit higher TPSA values than their corresponding aglycones because the sugar moiety introduces additional hydroxyl groups and other oxygen-containing functional groups, thereby increasing the polar surface area. Consequently, the relatively high TPSA of flavonoid glycosides may contribute to their lower passive membrane permeability and oral absorption compared with the corresponding aglycones.

- **Rotatable bonds:** A low number of rotatable bonds ensures that the molecule is more stable and has better bioavailability. According to Veber's rule, ≤ 10 is preferred.

- **P-glycoprotein (P-gp) substrate:** Molecules with a P-gp substrate can be pumped back into the lumen from the intestines. This situation reduces bioavailability. Therefore, being non-substrate provides an advantage.

- **Cytochrome P450 inhibition:** SwissADME; CYP1A2, CYP2C9, CYP2C19, CYP2D6, CYP3A4 predicts enzyme inhibition. The presence of numerous CYP inhibitors in natural products that can be used in kidney stone treatment is a disadvantage in terms of drug interactions.

- **PAINS and Brenk alerts:** Structures that may cause false positive biological activity in phenolic compounds are evaluated with PAINS filters. Brenk alerts, on the other hand, show functional groups that may cause potential toxicity. Both parameters are important in the selection of new drug candidates.

- **Lead-likeness:** Indicates whether natural compounds are drug candidates that can be optimized in the future. Phenolic compounds that meet the lead-likeness criteria are more suitable for structural optimization studies.

- **Synthetic accessibility:** Not directly related to biological activity. However, it is important in

terms of ease of industrial synthesis and cost of drug development.

Lipinski violations may be observed in most flavonoid glycosides due to their increased molecular weight. In contrast, aglycone forms (e.g., quercetin and kaempferol) generally exhibit more favorable pharmacokinetic properties [15-19].

RESULTS AND DISCUSSION

According to LC-HRMS analysis, naringenin was detected in the highest concentration among the flavonoids frequently reported in the literature for their potential to prevent kidney stone formation in *Helichrysum arenarium* extract, with an amount of 104,410 µg/g dry plant. In contrast, kaempferol and quercetin were found at lower levels, measured as 3,673 µg/g and 0,203 µg/g dry plant, respectively. Regarding phenolic compounds, caffeic acid was detected in the extract at a level of 6,248 µg/g dry plant, while chlorogenic acid was not detected. These results indicate that the extract is particularly rich in naringenin in terms of its kidney stone-preventing effect, while quercetin, kaempferol, and caffeic acid are present in lower concentrations.

SwissADME-predicted pharmacokinetic and drug-likeness properties of major flavonoids and phenolic compounds identified in *Helichrysum arenarium* is given in Table 1. Quercetin, kaempferol, and naringenin generally exhibit favorable absorption characteristics as kidney stone-preventing agents. The estimated human intestinal absorption (HIA) of all three flavonoids is over 70%, with naringenin showing the highest gastrointestinal absorption potential at 91.31%. This suggests that naringenin may have a higher rate of systemic circulation after oral administration and a higher probability of reaching kidney tissue. Quercetin (77.21%) and kaempferol (74.29%) also show acceptable levels of intestinal absorption. CaCO-2 permeability results also support these findings. In contrast, chlorogenic acid exhibited low gastrointestinal absorption, while caffeic acid showed a high absorption potential. Naringenin's log Papp value of 1.029 indicates a very high passive diffusion through the intestinal epithelium.

Table 1. SwissADME-predicted pharmacokinetic and drug-likeness properties of major flavonoids and phenolic compounds identified in *Helichrysum arenarium*

ADME property	Quercetin	Kaempferol	Naringenin	Chlorogenic acid	Caffeic acid
Gastro-intestinal absorption (GI)	High	High	High	Low	High
Bioavail-ability score	0.55	0.55	0.55	0.11	0.56
Lipinski rule of five	Yes; 0 violation	Yes; 0 violation	Yes; 0 violation	Yes; 1 violation (NH/OH>5)	Yes; 0 violation
Consensus logP	0.79	1.20	1.87	-0.71	0.92
ADME property	Quercetin	Kaempferol	Naringenin	Chlorogenic acid	Caffeic acid

Water solubility (ESOL)	-3.19 Soluble	-3.33 Soluble	-3.49 Soluble	-1.62 Very soluble	-1.89 Very soluble
TPSA	131.36	111.13	86.99	164.75	77.76
Rotatable bonds	1	1	1	5	2
P-gp substrate	No	No	Yes	No	No
PAINS	1 alert	0	0	1 alert	1 alert
Brenk	1 alert	0	0	2 alerts	2 alerts
Lead-likeness	Yes	Yes	Yes	No	No
Synthetic accessibility	3.23	3.14	3.01	4.16	1.81

Kaempferol's log P value of 0.032 suggests moderate permeability, while quercetin's log P value of -0.229 indicates relatively lower intestinal permeability. Therefore, naringenin can be considered advantageous in terms of bioavailability compared to the other two flavonoids. All three flavonoids have moderate water solubility, with log mol/L values ranging from -2.925 to -3.224. Quercetin has the highest solubility (-2.925 log mol/L), while naringenin shows the lowest solubility (-3.224 log mol/L). However, naringenin's high intestinal permeability can significantly offset the negative impact of its low solubility on bioavailability. In terms of water solubility, chlorogenic acid and caffeic acid are classified as "very soluble," while quercetin, kaempferol, and naringenin are classified as "soluble." The high solubility of chlorogenic acid and caffeic acid, in particular, can be considered an advantage, facilitating their transport in the urine and their interaction with calcium oxalate crystals. However, high solubility alone does not necessarily mean high bioavailability, as seen in the case of chlorogenic acid, which exhibits low gastrointestinal absorption. The fact that the skin permeability (log Kp) values are quite similar for the three compounds (approximately -2.74) indicates that they have similar characteristics in terms of transdermal application. However, since the primary route of administration in kidney stone treatment is oral, the clinical significance of this parameter is limited. The fact that all three flavonoids are predicted to be P-glycoprotein (P-gp) substrates suggests that they may be subject to P-gp-mediated efflux in the intestinal epithelium, which could somewhat reduce their bioavailability. However, the fact that none of them are predicted to be P-gp inhibitors (Type I or Type II) suggests that these compounds are unlikely to disrupt drug transport mechanisms by suppressing P-gp function, and may therefore offer an advantage in terms of drug interactions.

CONCLUSION

All three flavonoids exhibit favorable pharmacokinetic properties in terms of kidney stone prevention potential. However, naringenin stands

out as the most advantageous candidate in terms of oral bioavailability due to its highest HIA and CaCO₂ permeability. Quercetin and kaempferol, despite lower intestinal permeability, have acceptable absorption properties. When these pharmacokinetic findings are considered together with the antioxidant, anti-inflammatory, and calcium oxalate crystallization inhibitory biological effects reported in the literature, it suggests that naringenin, in particular, may have higher therapeutic potential for preventing kidney stone formation. However, these predictions need to be confirmed by crystallization experiments, cell culture studies, and *in vivo* models.

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