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Fatty-Acid-Dominant Phytochemistry, Divergent Bioactivity: A GC-MS Structure Activity Analysis of Four Medicinal Plant Extracts in Calcium Oxalate Inhibition and Cytotoxicity

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ABSTRACT

Background: Calcium oxalate urolithiasis remains one of the most common urological conditions worldwide, and plant extracts are routinely screened against its three crystallization stages nucleation, aggregation, and growth largely as isolated case studies rather than as a comparable set. **Objective:** This study asked whether four medicinal plant extracts that share a fatty-acid-dominant phytochemical matrix *Ammi visnaga*, *Carthamus tinctorius*, *Plantago ovata*, and *Eriobotrya japonica* also share a comparable pattern of calcium oxalate inhibition and toxicity, or whether their shared chemistry carries no predictive value for their biological behavior. **Methods:** Gas chromatography-mass spectrometry (GC-MS) compound-abundance profiles for the four extracts were reduced to six shared and marker compound classes and compared using Bray-Curtis dissimilarity. Bioactivity dissimilarity was computed from mean nucleation rate across four temperatures, zebrafish embryo viability at 900 µg/mL, and Vero cell half-maximal inhibitory concentration (IC_{50}), each min–max normalized. The two dissimilarity matrices were compared by Spearman rank correlation across the six unique extract pairs, and a thermal sensitivity index (percentage change in nucleation rate from room temperature to 40°C) was calculated for each extract. **Results:** Carthamus tinctorius was the most effective nucleation inhibitor at room temperature, 20°C, and 37°C (mean rate as low as 0.181 at 20°C) but lost the largest share of that activity by 40°C (+171.5%), while Ammi visnaga showed the smallest thermal loss (+4.8%) despite being the weakest nucleation inhibitor overall. Plantago ovata was the only cytotoxic extract of the four (Vero IC_{50} = 1.68 µg/mL; zebrafish viability 12.07% at 900 µg/mL), while the remaining three extracts were non-toxic by both assays. Chemical dissimilarity did not track with bioactivity dissimilarity across the six extract pairs (Spearman $\rho = -0.03$, $p = 0.96$); the pair with the most similar bulk fatty-acid chemistry (Ammi visnaga–Eriobotrya japonica, Bray-Curtis = 0.415) was not the pair with the most similar bioactivity, and Carthamus tinctorius–Plantago ovata combined near-identical fatty-acid profiles (Bray-Curtis = 0.414) with the largest bioactivity gap of any pair. **Conclusion:** Shared fatty-acid bulk chemistry does not predict shared calcium oxalate inhibitory or cytotoxic behavior among these four extracts; divergence appears instead to track with minor and marker phytochemicals methoxsalen, tannins, and the presence or absence of phenolics — rather than with the fatty-acid matrix common to all four.

المحتوى الكيميائي النباتي الغني بالأحماض الدهنية والأنشطة الحيوية المتباينة: دراسة تحليلية باستخدام GC-MS للارتباط بين البنية والفعالية لأربعة مستخلصات نباتية طبية ودورها في تثبيط أكسالات الكالسيوم والسمية الخلوية

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الملخص

الخلفية :(Background) لا يزال تحصي البول (urolithiasis) المرتبط بـ calcium oxalate أحد أكثر حالات المسالك البولية شيوعاً في جميع أنحاء العالم، وتُفحص المستخلصات النباتية بشكل روتيني ضد مراحل التبلور الثلاث الخاصة به — التنوي(nucleation) ، والتجمع(aggregation) ، والنمو — (growth) وذلك في الغالب كدراسات حالة معزولة وليس كمجموعة قابلة للمقارنة. الهدف :(Objective) تساءلت هذه الدراسة عما إذا كانت أربعة مستخلصات نباتية طبية تتشارك في مصفوفة كيميائية نباتية تغلب عليها الأحماض الدهنية وهي *Ammi visnaga*، و *Carthamus tinctorius*، و *Plantago ovata*، و *Eriobotrya japonica*تتشارك أيضاً في نمط مماثل من تثبيط calcium oxalate والسمية، أم أن كيمياءها المشتركة لا تحمل أي قيمة تنبؤية لسلوكها البيولوجي. المنهجية :(Methods) اختُزلت ملفات وفرة المركبات الناتجة عن تحليل GC-MS للمستخلصات الأربعة إلى ست فئات من المركبات المشتركة والمميزة، وقورنت باستخدام التباين بطريقة Bray-Curtis. حُسب تباين النشاط الحيوي من متوسط معدل التنوي (nucleation rate) عبر أربع درجات حرارة، وحيوية أجنة أسماك الزرد (zebrafish embryos) عند 900 ميكروجرام/مل، والتركيز المثبط النصف أقصى (IC_{50}) لخلايا Vero، حيث خضعت جميعها لتسوية الحد الأدنى والأقصى .(min–max normalized) قورنت مصفوفتا التباين بواسطة ارتباط الرتب لسبيرمان (Spearman rank correlation) عبر الأزواج الستة الفريدة للمستخلصات، وحُسب مؤشر الحساسية الحرارية (النسبة المئوية للتغير في معدل التنوي من درجة حرارة الغرفة إلى 40 درجة مئوية) لكل مستخلص. النتائج :(Results) كان مستخلص *Carthamus tinctorius* هو المثبط الأكثر فعالية للتنوي عند درجة حرارة الغرفة، و20 درجة مئوية، و37 درجة مئوية (متوسط معدل منخفض وصل إلى 0.181 عند 20 درجة مئوية) ولكنه فقد الحصة الأكبر من هذا النشاط عند 40 درجة مئوية (+171.5%)، بينما أظهر مستخلص *Ammi visnaga* أقل فقدان حراري (+4.8%) على الرغم من كونه أضعف مثبط للتنوي بشكل عام. كان مستخلص *Plantago ovata* هو المستخلص السام للخلايا الوحيد من بين الأربعة) قيمة IC_{50} لخلايا Vero = 1.68 ميكروجرام/مل؛ وحيوية أجنة أسماك الزرد 12.07% عند 900 ميكروجرام/مل(، بينما كانت المستخلصات الثلاثة المتبقية غير سامة وفقاً لكلا المقايستين. لم يتوافق التباين الكيميائي مع تباين النشاط الحيوي عبر الأزواج الستة للمستخلصات) معامل ارتباط سبيرمان$\rho = -0.03$ ، القيمة الاحتمالية $p = 0.96$؛ فالزوج الذي يتمتع بأكثر كيمياء كلية مشابهة للأحماض الدهنية (*Ammi visnaga–Eriobotrya japonica*، التباين بطريقة Bray-Curtis = 0.415) لم يكن الزوج الذي يتمتع بالنشاط الحيوي الأكثر تشابهاً، وجمع الزوج *Carthamus tinctorius–Plantago ovata* بين ملفات تعريف متطابقة تقريباً للأحماض الدهنية)التباين بطريقة Bray-Curtis = 0.414) مع أكبر فجوة في النشاط الحيوي مقارنة بأي زوج آخر. الخلاصة :(Conclusion)لا تتنبأ الكيمياء الكلية المشتركة للأحماض الدهنية بالسلوك المشترك لتثبيط calcium oxalate أو السمية الخلوية بين هذه المستخلصات الأربعة؛ وبدلاً من ذلك، يبدو أن التباين يتوافق مع المركبات الكيميائية النباتية الثانوية والمميزة — مثلmethoxsalen ، وtannins ، ووجود أو غياب — phenolics وليس مع مصفوفة الأحماض الدهنية المشتركة بين المستخلصات الأربعة جميعها.

الكلمات المفتاحية:

أوكسالات الكالسيوم

الحصاة البولية

الخلة البلدية

العصفر (القرطم)

القطونة الهندية

Introduction

Kidney stone disease placed a substantial and still-growing burden on health systems in 2021, when the Global Burden of Disease study attributed 106 million incident cases and a 34.5% rise in disability-adjusted life-years to urolithiasis since 2000, with calcium oxalate accounting for most stones (1). Calcium oxalate crystallization does not happen in one step; it proceeds through nucleation, aggregation, and crystal growth, and each stage can, in principle, be blocked independently, which is why in vitro screening assays measure all three rather than treating stone formation as a single event (2). Atmani and Khan established the spectrophotometric nucleation and aggregation assays still in routine use when they showed that an extract of Herniaria hirsuta altered calcium oxalate crystal number and size in human urine (3), and this assay framework has since been applied to dozens of individual plant extracts, including a direct demonstration that Ammi visnaga inhibits calcium oxalate monohydrate nucleation and aggregation in vitro (4). What this extract-by-extract literature has not asked is whether plants that converge on a similar bulk phytochemical matrix and fatty-acid-dominant profiles recur constantly across GC-MS screens of unrelated medicinal species also converge on a similar biological outcome, or whether that chemical similarity is simply uninformative about what the extract will do in a crystallization or toxicity assay. This study addresses that question directly for four extracts Ammi visnaga, Carthamus tinctorius, Plantago ovata, and Eriobotrya japonica reanalyzing their GC-MS compound profiles alongside nucleation, cytotoxicity, and embryotoxicity data (5) to test whether chemical resemblance among the four extracts predicts resemblance in biological behavior.

2. Materials and Methods

2.1 Plant material and extraction

The four plant materials analyzed in this study — Ammi visnaga (L.) Lam., Carthamus tinctorius L., Plantago ovata Forssk. (psyllium seed), and Eriobotrya japonica (Thunb.) Lindl. were sourced from a local market in Sebha, Libya, and taxonomically authenticated by botanists in the Department of Botany, Faculty of Science, Sebha University (5). Twenty grams of dried material from each plant was extracted by reflux in 500 mL distilled water for one hour, cooled, filtered through Whatman No. 1 filter paper (Cytiva, Marlborough, MA, USA), and dried in an oven at 42°C overnight; dry extracts were stored at 4°C until use.

2.2 Calcium oxalate nucleation, aggregation, and growth assays

Nucleation inhibition was assayed following Atmani and Khan (3): calcium chloride (5 mmol/L) and sodium oxalate (7.5 mmol/L) solutions were prepared in Tris-NaCl buffer (0.05 mol/L Tris, 0.15 mol/L NaCl, pH 6.5; Sigma-Aldrich, St. Louis, MO, USA), combined with 2 mg/mL extract, and monitored at 620 nm at 30 and 60 minutes at room temperature, 20°C, 37°C, and 40°C, in triplicate, with citric acid as the positive control. Aggregation inhibition used pre-formed calcium oxalate crystals (0.8 mg/mL) in Tris-HCl/NaCl buffer, read at 620 nm after 30 minutes at 37°C. Crystal growth inhibition followed a spectrophotometric depletion assay monitoring free oxalate consumption at 214 nm over 600 seconds, with cystone or extract (0.5–1.0 mg/mL) added to a calcium oxalate monohydrate crystal slurry in Tris-HCl buffer (pH 7.2). Percentage inhibition for each assay was calculated as $(1 - \text{ODtest}/\text{ODcontrol}) \times 100$.

2.3 Qualitative phytochemical screening

Alkaloids, flavonoids, phenolic compounds, tannins, and phytosterols were screened by standard colorimetric spot tests (picric acid, lead acetate, ferric chloride, sodium hydroxide emulsification, and the chloroform/sodium hydroxide ring test, respectively), scored as present or absent for each extract.

2.4 GC-MS analysis

Dried extracts were dissolved in dimethyl sulfoxide (DMSO; Sigma-Aldrich) and analyzed on a Thermo Scientific™ TRACE™ 1310 GC system coupled to a mass spectrometer operated in electron-impact ionization, full-scan mode, with splitless injection and a non-polar capillary column. Compounds were identified against the NIST/Wiley spectral library using Thermo Scientific™ Xcalibur™ software, and relative abundance was calculated from chromatographic peak area (Area %).

2.5 Zebrafish embryo toxicity assay

Extract toxicity was evaluated in zebrafish (Danio rerio) embryos following the principles of OECD Test Guideline 236 (6). Extracts were diluted in DMSO to 200, 500, and 900 µg/mL. Embryos at the 4–8 cell stage were exposed in individual wells of 24-well plates and incubated at 28.5°C; viability (normal heart rate, absence of morphological abnormality, spontaneous movement) was assessed daily to 96 hours post-fertilization and expressed as a percentage of the DMSO-only control, in triplicate.

2.6 Vero cell culture and SRB cytotoxicity assay

Vero (African green monkey kidney) cells (Capricorn Scientific, Ebsdorfergrund, Germany, supplied via a regional distributor in Egypt) were maintained in DMEM (Gibco, Thermo Fisher Scientific, Waltham, MA, USA) with 10% heat-inactivated fetal bovine serum, 100 U/mL penicillin, and 100 µg/mL streptomycin at 37°C in 5% CO_2. Cytotoxicity was assessed by the sulforhodamine B (SRB) assay of Skehan et al. (7): cells were seeded at 5×10^3 per well, exposed to extract for 24 hours, fixed with 10% trichloroacetic acid, stained with 0.4% (w/v) SRB, and read at 540 nm on an Infinite F50 microplate reader (Tecan, Männedorf, Switzerland) to derive IC_{50} values.

2.7 Statistical analysis

Descriptive statistics and one- and two-way ANOVA for the nucleation, aggregation, growth, and toxicity assays were performed in GraphPad Prism (version 9.0, GraphPad Software, San Diego, CA, USA) on triplicate data, with $p < 0.05$ considered significant. For the structure-activity analysis reported here, GC-MS compound-abundance data were reduced to six shared or marker compound classes per extract (trilinolein; hexadecanoic acid; oleic acid/cis-13-eicosenoic acid; cis-vaccenic acid; triolein; and one plant-specific marker compound), and pairwise Bray-Curtis dissimilarity was computed across the four chemical profiles, following the chemotaxonomic clustering approach used for GC-MS lipophilic-compound profiling in other plant systems (8). Bioactivity dissimilarity was computed as the normalized Euclidean distance across three endpoints mean nucleation rate averaged over the four assay temperatures, zebrafish viability at 900 µg/mL, and Vero IC_{50} after min–max normalization. The two dissimilarity matrices were compared by Spearman rank correlation across the six unique extract pairs. A thermal sensitivity index was calculated for each extract as the percentage change in mean nucleation rate between room temperature and 40°C. All chemometric calculations were performed in Python 3 (SciPy 1.x, pandas).

3. Results

3.1 Nucleation inhibition across temperatures

Mean nucleation rate where a lower rate indicates stronger inhibition varied significantly by extract at every temperature tested ($p < 0.0001$ at room temperature and 20°C; $p < 0.00001$ at 37°C and 40°C; Table 1). Carthamus tinctorius had the lowest mean nucleation rate at room temperature (0.214), 20°C (0.181), and 37°C (0.189), but its rate rose to 0.581 ± 0.462 at 40°C. Ammi visnaga had the highest mean nucleation rate at all four temperatures (1.123 ± 0.369 at room temperature, 1.027 ± 0.139 at 20°C, 0.841 ± 0.081 at 37°C, and 1.177 ± 0.220 at 40°C). Plantago ovata showed the highest single value recorded in the dataset (1.715 at 20°C) and remained above 1.0 at every other temperature. Eriobotrya japonica held an intermediate position at each temperature (0.307–0.661). A significant plant-by-temperature interaction was found ($p < 0.00001$).

Extract	Room temp.	20°C	37°C	40°C
Ammi visnaga	1.123 ± 0.369	1.027 ± 0.139	0.841 ± 0.081	1.177 ± 0.220
Carthamus tinctorius	0.214	0.181	0.189	0.581 ± 0.462
Plantago ovata	1.086	1.715	1.146	1.364
Eriobotrya japonica	0.327	0.307	0.370	0.661

Table 1. *Mean calcium oxalate nucleation rate (± SD where available) for each extract at four assay temperatures. Lower values indicate stronger nucleation inhibition.*

3.2 Thermal sensitivity of nucleation inhibition

The percentage change in mean nucleation rate between room temperature and 40°C was +171.5% for Carthamus tinctorius, +102.1% for Eriobotrya japonica, +25.6% for Plantago ovata, and +4.8% for Ammi visnaga (Figure 1, Table 2).

Extract	Thermal sensitivity index (%)
Ammi visnaga	+4.8
Plantago ovata	+25.6
Eriobotrya japonica	+102.1
Carthamus tinctorius	+171.5

Table 2. *Thermal sensitivity index (percentage increase in mean nucleation rate from room temperature to 40°C) for each extract, ranked from most to least thermally stable.*

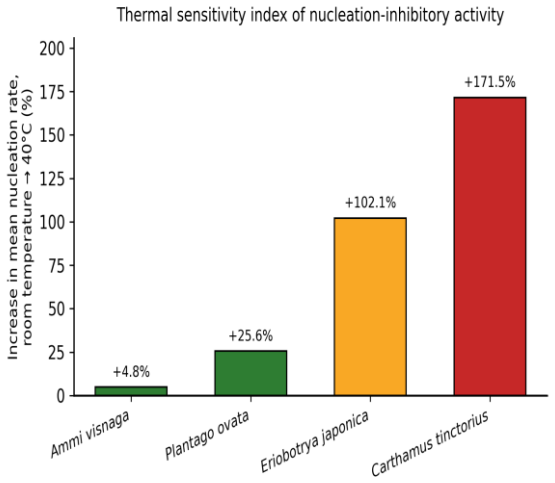


Figure 1. *Thermal sensitivity index of calcium oxalate nucleation-inhibitory activity for the four extracts.*

3.3 Cytotoxicity and embryotoxicity

Vero cell IC_{50} was 1.68 µg/mL for Plantago ovata, reducing viability to approximately 22.9% at 500 µg/mL; the other three extracts had IC_{50} values exceeding 500 µg/mL (Table 3). Zebrafish embryo viability at 900 µg/mL was 12.07% for Plantago ovata ($p < 0.00001$ versus control), 74.14% for Ammi visnaga ($p = 0.030$), 86.21% for Eriobotrya japonica ($p = 0.160$), and 93.10% for Carthamus tinctorius

(p = 0.398) (Table 3).

Extract	Vero IC_{50} (µg/mL)	Zebrafish viability at 900 µg/mL (%)	p-value
Ammi visnaga	> 500	74.14	0.030
Carthamus tinctorius	> 500	93.10	0.398
Plantago ovata	1.68	12.07	< 0.00001
Eriobotrya japonica	> 500	86.21	0.160

Table 3*. Vero cell IC_{50} (SRB assay) and zebrafish embryo viability at the highest tested concentration (900 µg/mL) for each extract, with the p-value for the comparison against the DMSO-only control.*

3.4 GC-MS compound-class profiles

Reduction of the four GC-MS profiles to six shared or marker compound classes showed trilinolein present in all four extracts (6.6–13.3 Area %), hexadecanoic acid present in three of four extracts (absent in Plantago ovata), and cis-vaccenic acid restricted to Eriobotrya japonica alone (26.6 Area %), with each extract also carrying one distinguishing marker compound: methoxsalen in Ammi visnaga (15.7%), erucic acid in Carthamus tinctorius (5.0%), a silylated palmitic acid derivative in Plantago ovata (6.2%), and 1,5,9-decatriene-2,3,5,8-tetramethyl in Eriobotrya japonica (15.2%) (Table 4, Figure 2).

Compound class	Ammi visnaga	Carthamus tinctorius	Plantago ovata	Eriobotrya japonica
Trilinolein	10.08	10.09	13.29	6.59
Hexadecanoic acid	7.23	17.09	—	11.13
Oleic acid / cis-13-eicosenoic acid	—	24.06	9.21	—
cis-Vaccenic acid	—	—	—	26.61
Triolein	—	6.34	13.29	6.59
Marker compound (unique)	15.71	5.00	6.18	15.18

Table 4. *GC-MS compound-class abundance matrix (Area %) for the four extracts. Dashes indicate the compound class was not detected.*

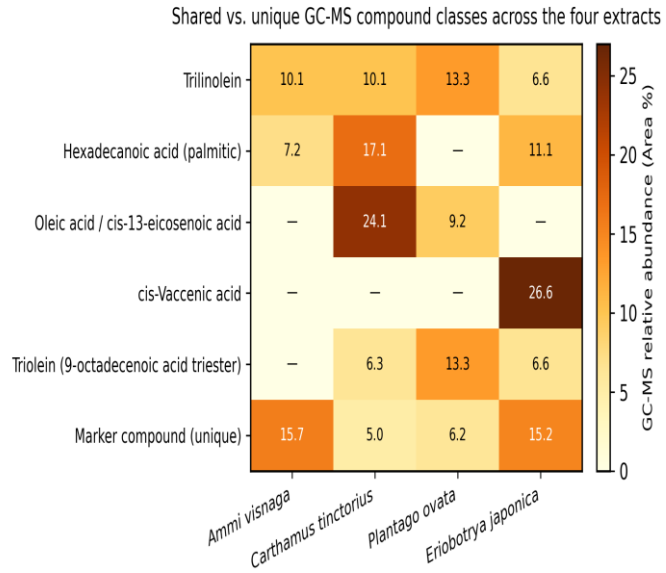


Figure 2. *Heatmap of shared and unique GC-MS compound classes across the four extracts (Area %).*

3.5 Chemical dissimilarity versus bioactivity dissimilarity

Pairwise Bray-Curtis dissimilarity of the six-class GC-MS profiles ranged from 0.414 (Carthamus tinctorius–Plantago ovata) to 0.642 (Plantago ovata–Eriobotrya japonica) (Table 5). Bioactivity dissimilarity, computed from mean nucleation rate, zebrafish viability at 900 µg/mL, and Vero IC_{50}, ranged from 0.148 (Carthamus tinctorius–Eriobotrya japonica) to 1.732 (Carthamus tinctorius–Plantago ovata) (Table 5). Spearman correlation between chemical dissimilarity and bioactivity dissimilarity across the six extract pairs

Extract pair	Chemical dissimilarity (Bray-Curtis)	Bioactivity dissimilarity (Euclidean)
Ammi visnaga – Carthamus tinctorius	0.533	0.761
Ammi visnaga – Plantago ovata	0.566	1.289
Ammi visnaga – Eriobotrya japonica	0.415	0.622
Carthamus tinctorius – Plantago ovata	0.414	1.732
Carthamus tinctorius – Eriobotrya japonica	0.548	0.148
Plantago ovata – Eriobotrya japonica	0.642	1.616

Table 5. *Pairwise chemical (GC-MS profile) dissimilarity and bioactivity dissimilarity across the six unique extract pairs. Spearman $\rho = -0.03$, $p = 0.96$ ($n = 6$ pairs).*

4. Discussion

This study set out to test whether four medicinal plant extracts that converge on a common fatty-acid-dominant phytochemical matrix also converge on a common pattern of calcium oxalate inhibitory and cytotoxic behavior, and the pairwise comparison answers that question directly: they do not. Carthamus tinctorius and Plantago ovata returned almost the same Bray-Curtis chemical dissimilarity as Ammi visnaga and Eriobotrya japonica (0.414 versus 0.415), yet the first pair produced the widest bioactivity gap of any comparison in the dataset while the second produced a comparatively modest one. If bulk fatty-acid content were driving these extracts' effects on nucleation and cell viability, chemically similar pairs should behave similarly; instead, Carthamus tinctorius remained a non-toxic, temperature-sensitive nucleation inhibitor while Plantago ovata, with an almost identical oleic acid/hexadecanoic acid/trilinolein backbone, was the only cytotoxic extract of the four. That pattern points toward the minor and marker compounds methoxsalen, tannins, the presence or absence of phenolics rather than the shared fatty-acid matrix as the more plausible drivers of the divergence, a reading consistent with chemotaxonomic work in other plant systems showing that overall metabolite similarity and specific bioactive signatures do not always track together even within a single population (8).

The temperature response of Carthamus tinctorius is the second finding worth taking on its own terms. Its nucleation inhibition was the strongest of the four extracts at room temperature, 20°C, and 37°C, but that advantage collapsed by 40°C, a +171.5% rise in mean nucleation rate far larger than any other extract's thermal loss. Safflower's principal chemistry oleic acid and hexadecanoic acid dominate its GC-MS profile here, consistent with the fatty-acid-rich composition reported for safflower achenes generally (9) is not obviously heat-labile at the molecular level, which suggests the loss of activity more likely reflects a change in how the extract's minor inhibitory constituents interact with the crystallization interface at higher temperature rather than thermal degradation of the bulk matrix itself; distinguishing between those two explanations would require isolating and re-testing the extract's individual fractions across the same temperature range. In practical terms, an extract this sensitive to a nine-to-fifteen-degree temperature shift would need a stabilizing formulation strategy before any nutraceutical development, since physiological and mildly febrile conditions sit within the range where its advantage disappears.

Ammi visnaga showed the opposite profile: the weakest nucleation inhibition of the four extracts at every temperature, paired with the smallest thermal sensitivity. Methoxsalen, the extract's dominant GC-MS marker here at 15.7 Area %, is a furanocoumarin with a pharmacology well outside stone disease it is best known for

phototherapy but Ammi visnaga's furanocoumarins and furanochromones (khellin and visnagin in particular) have separately been shown to protect renal epithelial cells against oxalate-induced damage (10), which offers a more specific mechanistic account for a strong aggregation effect than "flavonoid content" would. The extract's IC_{50} exceeding 500 µg/mL in the Vero assay is consistent with the acute and subacute toxicity profile reported for Ammi visnaga in rats, where standard doses produced no marked organ toxicity (11), though the zebrafish assay here did detect a significant viability drop at the highest concentration tested (900 µg/mL, $p = 0.030$) that the Vero assay did not, a discrepancy between the two toxicity models worth flagging rather than smoothing over.

Plantago ovata is the extract whose result most clearly resists a shared-chemistry explanation. Its GC-MS profile overlaps substantially with Carthamus tinctorius and Eriobotrya japonica in trilinolein and triolein content, yet it was the only one of the four to show meaningful cytotoxicity in either toxicity assay, and by a wide margin (Vero IC_{50} 1.68 µg/mL against IC_{50} values exceeding 500 µg/mL for the other three). Psyllium seed's fiber and mucilage content is its best-known pharmacological feature, but Plantago seed and leaf extracts have independently shown Caco-2 antiproliferative activity at IC_{50} values in a broadly similar low-hundreds-of-µg/mL range (12), and selective cytotoxicity against breast carcinoma lines has been reported for a related Plantago species, tentatively attributed to flavonoid content. Tannins were detected in this study's Plantago ovata extract but in none of the other three, a compositional feature distinct from the shared fatty-acid backbone the four extracts otherwise hold in common. More broadly, unsaturated and hydroxylated fatty acid derivatives are known to promote apoptosis through oxidative and mitochondrial pathways in a manner that depends heavily on the specific compound and cellular context rather than on fatty acid content in the aggregate (13), which is consistent with a marker compound tannins, in this case outweighing the bulk lipid profile as the more plausible driver of this extract's distinct cytotoxicity. None of this identifies the active cytotoxic principle with certainty, but it reframes the finding usefully: an extract already known as a food-grade fiber supplement showing this degree of selective cytotoxicity is a signal worth isolating and re-testing as a candidate antiproliferative fraction rather than a toxicity result to be explained away.

This analysis carries two limitations that bear directly on how far the structure-activity claim can be pushed. The chemical and bioactivity dissimilarity matrices rest on four extracts, giving only six unique pairwise comparisons, which is too few for the Spearman correlation to reach conventional statistical significance regardless of the true underlying relationship; the $\rho = -0.03$ result should be read as a pattern consistent with no relationship rather than as statistical proof that none exists. The GC-MS compound-class reduction also collapses several distinct triglyceride and fatty-acid-ester isomers into shared categories for comparability across extracts, which could mask genuine differences in the exact fatty-acid composition that a targeted lipidomic re-analysis would resolve. The logical next step is isolating the specific marker fractions identified here the methoxsalen-containing fraction of Ammi visnaga, the tannin fraction of Plantago ovata and re-testing them individually against the same nucleation, aggregation, and cytotoxicity assays, which would establish directly whether these minor components, rather than the shared fatty-acid backbone, are in fact responsible for the divergence this analysis has only been able to demonstrate indirectly.

5. Conclusion

Four medicinal plant extracts that share a fatty-acid-dominant GC-MS profile do not share a comparable pattern of calcium oxalate inhibitory or cytotoxic activity: chemical similarity and bioactivity similarity were uncorrelated across all six pairwise comparisons ($\rho = -0.03$). Carthamus tinctorius combined the strongest nucleation inhibition of the four extracts with the sharpest loss of that activity at 40°C, Ammi visnaga combined the weakest nucleation inhibition with the greatest thermal stability, and Plantago ovata was the only extract of the four to show meaningful cytotoxicity despite a fatty-acid backbone similar to the other three. These results argue against treating bulk phytochemical similarity as a proxy for expected bioactivity in plant-derived anti-urolithiatic screening, and support isolating each extract's minor and marker compounds for direct mechanistic testing before drawing conclusions from group-level chemical resemblance alone.

Author Contributions

F.A : Methodology S.S.S : Conceptualization and supervision, , investigation, formal analysis, and writing – original draft, , T.A : Proofread All authors read and approved the final manuscript.]

Conflict of Interest

The authors declare no conflict of interest.

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Data Availability

The GC-MS, nucleation, toxicity, and derived chemometric data supporting this analysis are available from the corresponding author on request.

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