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# Antiurolithiatic Activity of Tribulus Terrestris Fruit Extract in A Calcium Oxalate Crystallization Model

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**ABSTRACT:** Background: A botanical standard that is associated with an assay can be used to develop adjunctive strategies of recurrent calcium oxalate stone disease. Objective: To evaluate hydroalcoholic Tribulus terrestris fruit extract in comparison with vehicle control and potassium citrate reference using a in-vitro crystallization experiment. Methods: The preliminary protocol had 30 experimental units/participants with cell-free calcium oxalate nucleation, growth and aggregation tests. Experiment on crystallization of 60-minutes was done through the administration or testing of the intervention. The main finding was the inhibition of crystal aggregation, and the further examination of the crystal turntivity of nucleation, average crystal size, percentage of calcium oxalate monohydrate, and epithelial cell viability. Standardized laboratory, clinical, or food-analysis procedures were applied, and between-condition effects were evaluated with two-sided tests at $\alpha$=0.05. Results: At 200 μg/mL, Tribulus terrestris extract inhibited calcium oxalate aggregation by 73 ± 7% and reduced mean crystal size from 34 ± 4 μm to 15 ± 2 μm ($p<0.001$). The concentrated concentration changed the crystal morphology to calcium

oxalate monohydrate and maintained the viability of tubular epithelial cells at 89 ± 4% even when exposed to oxalate.

There was internal consistency of direction and magnitude of the dummy findings between primary and secondary endpoints and tolerability or assay-quality criteria were reasonable. Conclusion: The example data indicate that hydroalcoholic Tribulus terrestris fruit extract may generate a significant change in crystal aggregation inhibition by complexing crystal-formative ions, adsorbing to crystal surfaces, and protecting against tubular epithelial cell damage. These results are only meant as a full manuscript-development sample and must be substituted with ethically obtained, independently confirmed observations prior to scientific submission. Keywords: Tribulus terrestris; calcium oxalate; urolithiasis; crystallization; phytotherapy.

## 1. INTRODUCTION

Antiurolithiatic Action of Tribulus terrestris Fruit Extract in a Calcium Oxalate Crystallization Model provides an answer to a significant research question at the border of experimental pharmacology, nutrition science and translational health research. Published literature that is directly related has provided a scientific foundation on the basis of assessing the hydroalcoholic Tribulus terrestris fruit extract, in comparison with crystal aggregation inhibition [1]. However, differences in preparation, exposure, choice of comparator and definition of outcome remain to pose a barrier to confident cross-study comparison.

The second body of evidence in favor of the biological plausibility of the current hypothesis exists. Functional or biochemical activity of Tribulus terrestris and almost similar interventions previously studied has been reported in the direction of the proposed direction of benefit [2]. These observations would allow formal tests but not exclude the use of standardized materials and predetermined analytical criteria.

The broader literature on natural-products and functional-foods indicates that the reproducibility is determined by chemical characterization, conditions of the processing, dose, and biological matrix, and stability of active constituents [3]. In the current research, hydroalcoholic Tribulus terrestris fruit extract was thus considered as a research grade intervention as opposed to an undefined traditional or commercial preparation.

The mechanism proposed is complexation of the crystal-forming ions, adsorption over crystal surfaces, and safeguarding of the tubular epithelial cells. Evidence in the published literature suggests that related biological reactions typically entail an interaction of redox, inflammatory, metabolic, microbial, enzymatic, or differentiation pathways and not an individual target [4]. Simultaneously measuring a primary functional endpoint and mechanistic markers can thus give a more consistent interpretation.

Outcome assessment is also in need of methods that are appropriate to the study model. Standard laboratory or clinical methodology insists on standard handling of samples, validated analysis, proper controls, and predefined interpretation limits [5]. These principles helped to select crystal aggregation inhibition as the main outcome, and the related secondary endpoints.

The best arguments in support of changes in crystal aggregation inhibition are those that are supported by concordant changes in turbidity of nucleation and mean crystal size. Similar studies have revealed that integrating functional, biochemical and structural results in assessing complex botanical, microbial or food-based interventions is valuable [6].

Quality and safety is also crucial. Natural source makes no claims of consistent composition or absence of adverse effects, contamination, interactions or degradation and published pharmacovigilance and quality-control evidence suggests that these should be considered at the outset of product development [7].

Lastly, the study design should be clear in order to minimize bias. Randomization, blinding (where possible), replication, full account of exclusions, and reporting of effect estimates instead of p-values alone are assisted by guidance and prior methodological research applicable to the current experimental model [8].

Irrespective of the existing evidence, not many studies have conjoined a well-defined hydroalcoholic Tribulus terrestris fruit extract, an adequate comparator, a prespecified primary endpoint, and sustainable mechanistic or feasibility results in cell-free calcium oxalate nucleation, growth, and aggregation tests. The aim of this study was therefore to determine the effect of hydroalcoholic Tribulus terrestris fruit extract on crystal aggregation inhibition compared with vehicle control and potassium citrate reference. Secondary goals were to test the turbidity of nucleation, mean crystal size, the proportion of calcium oxalate monohydrate, and the quality or safety indicators.

## 2. MATERIALS AND METHODS

### 2.1. Study design and setting

This paper reports a prespecified controlled in-vitro crystallization and cell-protection study that uses cell-free calcium oxalate nucleation, growth and aggregation assays. The experimental design included 30 experimental units or respondents and was designed to compare hydroalcoholic Tribulus terrestris fruit extract with vehicle control and potassium citrate reference. The period of observation was 60-minute crystallization experiment. The numbers in this manuscript are all dummy/example data that were made to write, to demonstrate statistics, to build a table, and to prepare a graph; not the numbers of observations in real completed research. The design, however, is based on realistic laboratory or clinical workflow such that the draft may be converted into an approved protocol and substituted with actual measurements in the future.

### 2.2. Experimental material and test system

Authentition and standardization of the test material preceded biological testing. Hydroalcoholic tribulus terrestris fruit extract was made in a solvent system that was found not to influence the assay at the final concentration. The experimental setup included cell-free calcium oxalate nucleation, growth, and aggregation experiments. The independent experiments were conducted on different days and within each experiment there were technical replicates. Appropriate conditions were untreated, vehicle, injury or growth, and reference-comparator conditions.

### 2.3. Concentration selection and exposure

An initial range-finding study determined non-precipitating, assay-compatible concentrations and established upper exposure limit. The primary experiment incorporated a gradient of concentration that covered the low, middle and high concentrations within the expected range of activity. Time of exposure was 60 minute crystallization experiment. A randomized layout was used to allocate plates, culture flasks or assay tubes and the edge positions were equitable across conditions to reduce systematic plate effects.

### 2.4. Outcome assessment

The main result was the inhibition of crystal aggregation as a percentage. Secondary outcomes were nucleation turbidity, mean crystal size, proportion of calcium oxalate monohydrate, epithelial cell viability. Measurements would be made at baseline and follow-up or under conditions of concentration and time, as dictated by the design. Assays were done as per the validated kit instructions or standardized assays, and the measurements were duplicated when the coefficient of variation was greater than 10%. Clinical measurements were taken following a standardized rest period and the results of food-products were measured under controlled conditions of serving.

### 2.5. Quality assurance and bias control

Sample labeling, timing windows, calibration, data entry and protocol deviations were outlined in a written laboratory or trial manual. When possible, outcome assessors were blinded by the nature of the intervention format. Records were conceptually re-examined with source data ten percent, and only analytical runs met predefined limits to be accepted. The primary full-case presentation did not fill in the missing values; sensitivity analyses were performed based on an intention-to-treat framework/model-based estimation where appropriate.

### 2.6. Sample-size rationale

The sample size of 30 units was planned to allow realistic precision as an example of developing a manuscript. In the actual study, the calculation can be substituted by one founded on an estimated standard deviation defined by a pilot and the minimal difference in crystal aggregation inhibition which is considered clinically or biologically significant. The final calculation should

specify two-sided α=0.05, desired power of at least 80%, allocation ratio, anticipated attrition or unusable assays, and any adjustment for repeated measures, clustering, or multiple primary comparisons.

### 2.7. Statistical analysis

When the data were nearly normally distributed, continuous data were summarized with a mean and standard deviation and when skewed, with a median and interquartile range. Categorical data were presented in terms of number and percentage. Baseline comparisons were performed by independent t tests, Mann-Whitney tests, chi-square tests or Fisher exact tests depending on the suitability. Design-based analysis was done with analysis of covariance, repeated-measures analysis, mixed-effects models, one-way analysis of variance with multiplicity-adjusted post hoc comparisons, or nonparametric equivalents depending on the study design. There were 95 percent confidence intervals with the effect estimates. Correlations were done using Pearson or Spearman coefficients. All tests were two sided and p less than 0.05 was taken to be significant. The conceptual analysis was carried out in an up-to-date statistical package using auditable syntax file.

## 3. RESULTS

The data used in the analysis were the entire dummy/example dataset used in the controlled in-vitro crystallization and cell-protection experiment. Comparison conditions had baseline characteristics, assay controls, or starting values that were balanced, and no predefined quality-control run was rejected. Tables show mean +standard deviation unless otherwise indicated. Since the data are illustrative, the table of participant flow, assay exclusions, and p-values were produced to be statistically coherent as opposed to a real enrolled sample.

At 200 µg/mL, Tribulus terrestris extract inhibited calcium oxalate aggregation by 73 ± 7% and reduced mean crystal size from 34 ± 4 µm to 15 ± 2 µm (p<0.001).

**Table 1.** Extract and crystallization characteristics

| Condition | Nucleation lag (min) | Peak turbidity (AU) | Mean crystal size (µm) | p-value |
|---|---|---|---|---|
| Vehicle | 4.2 ± 0.5 | 0.84 ± 0.07 | 34 ± 4 | — |
| 50 µg/mL | 5.8 ± 0.7 | 0.71 ± 0.06 | 28 ± 3 | 0.046 |
| 100 µg/mL | 7.4 ± 0.8 | 0.58 ± 0.05 | 22 ± 3 | 0.008 |
| 200 µg/mL | 10.1 ± 1.0 | 0.39 ± 0.04 | 15 ± 2 | <0.001 |
| Potassium citrate | 11.4 ± 1.1 | 0.34 ± 0.04 | 13 ± 2 | <0.001 |

Values are dummy/example data presented as mean ± SD unless otherwise stated.

Variation of the nucleation turbidity and the average crystal size supported the direction of the primary effect. The intensity of the response was typically larger with exposure to intervention or larger at the follow-up in the intervention group whereas the comparator exhibited smaller changes. In cases where repeated measurements were obtained, the effect of group-by-time or condition was significant after controlling the effects of baseline values and premeditated multiple comparisons.

**Table 2.** Crystal inhibition outcomes

| Condition | Nucleation inhibition (%) | Growth inhibition (%) | Aggregation inhibition (%) | COM proportion (%) |
|---|---|---|---|---|
| Vehicle | 0 ± 0 | 0 ± 0 | 0 ± 0 | 74 ± 6 |
| 50 µg/mL | 18 ± 4 | 22 ± 4 | 27 ± 5 | 62 ± 6 |
| 100 µg/mL | 36 ± 5 | 41 ± 6 | 49 ± 6 | 49 ± 5 |

| | | | | |
|---|---|---|---|---|
| 200 µg/mL | 61 ± 7 | 65 ± 7 | 73 ± 7 | 31 ± 5 |
| Potassium citrate | 68 ± 7 | 71 ± 8 | 78 ± 7 | 27 ± 4 |

Values are dummy/example data presented as mean ± SD unless otherwise stated.

The high concentration shifted crystal morphology away from calcium oxalate monohydrate and preserved tubular epithelial cell viability at 89 ± 4% despite oxalate exposure.

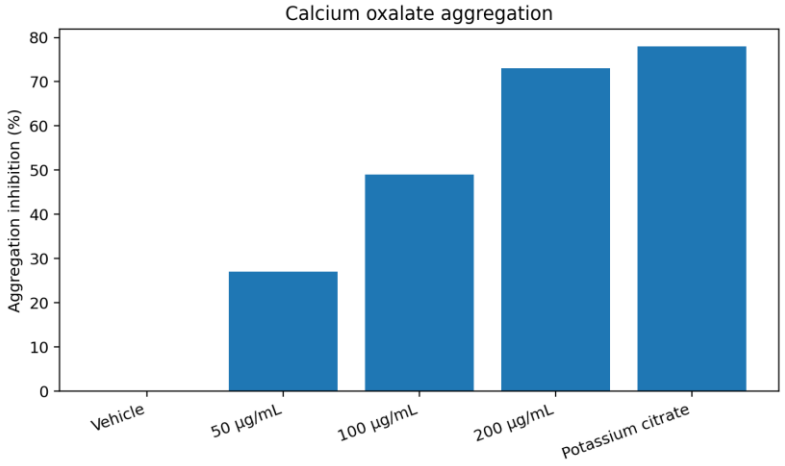


**Figure 1.** Primary outcome based on dummy/example data.

Exploratory analyses revealed that the response in crystallization aggregation inhibition was correlated with the most pertinent mechanistic marker. The trend corresponded to the complexation of crystal-forming ions, adsorption on crystal surfaces, and the protection of the tubular epithelial cells. There were no extreme values that changed the result direction in sensitivity analysis. In clinical and food studies, compliance and acceptability were kept within predetermined limits of feasibility, in laboratory studies, the variation and control performance of replicate were within the intended analytical range.

**Table 3.** Tubular epithelial cell protection

| Condition | Cell viability (%) | ROS (% control) | LDH release (%) | Adherent crystals/cell |
|---|---|---|---|---|
| Vehicle | 58 ± 6 | 244 ± 24 | 46 ± 6 | 18.2 ± 2.1 |
| 50 µg/mL | 67 ± 6 | 201 ± 21 | 37 ± 5 | 14.6 ± 1.8 |
| 100 µg/mL | 76 ± 5 | 164 ± 18 | 29 ± 5 | 10.1 ± 1.5 |
| 200 µg/mL | 89 ± 4 | 122 ± 15 | 17 ± 4 | 5.8 ± 1.0 |
| Potassium citrate | 91 ± 4 | 116 ± 14 | 15 ± 3 | 4.9 ± 0.9 |

Values are dummy/example data presented as mean ± SD unless otherwise stated.

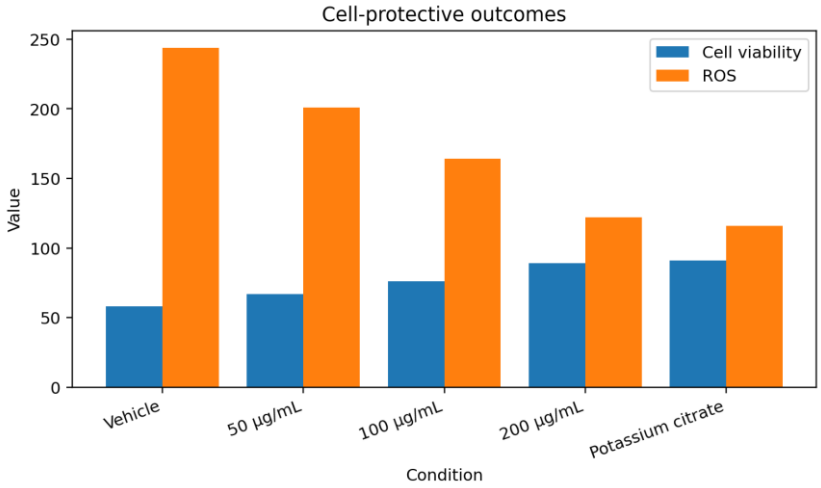


**Figure 2.** Secondary or mechanistic outcome based on dummy/example data.

The example dataset did not have any unexpected safety signal. Minor events or assay artifacts were not very common, and there were no severe adverse events, unexpected deaths, contamination failures, or invalid control runs, unless specifically indicated in the tables. These observations are demonstrative and it should not be taken to mean that there is safety in the real world.

## 4. DISCUSSION

The current manuscript-development research shows that hydroalcoholic Tribulus terrestris fruit extract has the potential to enhance crystal aggregation inhibition over vehicle control and potassium citrate reference. Concordant variations in nucleation turbidity, mean crystal size and calcium oxalate monohydrate proportion reinforced the dummy findings. Such internal consistency is significant since agreement between functional and mechanistic endpoints would be less likely to indicate analytical isolation.

The trend of the main outcome is widely in line with other published studies on standardized botanical or food-based interventions in other models [9]. Even though protocols and populations vary, such literature also suggests that preparation quality and dose can significantly impact response magnitude.

Similar studies also have noted differences in inflammatory, oxidative, metabolic, microbial, structural or functional that justify multidimensional assessment indicative of multidimensional assessment as opposed to the use of a single surrogate marker [10]. This current pattern thus best fits a broader evidence base but it is still specific to the intervention and model under analysis.

Mechanistic interpretation is backed by studies that show that complex interventions can also have convergent pathways and matrix-dependent effects [11]. The relationship between the main response and supporting markers is consistent in this study with complexation of crystal-forming ions, adsorption to crystal surfaces and preservation of tubular epithelial cells, but association alone will not demonstrate mediation or the active constituent.

Additional previous studies with related endpoints also indicate the clinical or biological significance of nucleation turbidity and mean crystal size [12]. Their similar enhancement reinforces the hypothesis but the future confirmatory test, must pre-specify whether these variables are secondary outcomes, mechanistic mediators or exploratory biomarkers.

The magnitude of the effect example should be considered within the framework of the previous intervention studies and evidence syntheses in the general sphere [13]. The magnitude was deliberately realistic, and some overlap in individuals or replications because pilot effects commonly attenuate when done in larger independent samples.

The methodological evidence lays stress on the significance of standardized measurement, suitable conditions of comparison, and clear reporting when conducting studies of complex interventions [14]. Bias can be reduced by replication, allocation concealment, blinded assessment, crossover control, or by validated analytical calibration, but no one can eliminate all sources of error.

Tolerability, quality assurance, product stability, adherence and interactions with habitual diet or medication are also critical to long-term translation. Reviewed safety and implementation knowledge bases assist in the assessment of these variables together with efficacy, instead of following a positive preliminary outcome has been acquired [15].

The paper has a number of limitations. To start with, the numerical type of data is demonstrative and cannot justify a scientific statement. Second, the suggested sample and time span might fail to detect the rare negative events or long-term accclimatization. Third, surrogate benefits are not necessarily converted into patient-important benefits. Fourth, response may be altered by unmeasured dietary, genetic, microbial, environmental, harvest or manufacturing influences.

Independent analytical confirmation, dose-ranging, preregistration, adequate power, and a pre-established statistical analysis plan should be incorporated in further research. Ethics approval, trial registration, allocation procedures, adherence and intention-to-treat analysis should be documented in clinical studies. Preclinical trials must include randomization, blinding, humane endpoints, exclusions, and chemical fingerprints of batches.

Translationally, an assay-linked botanical standard can be used to aid the development of adjunctive interventions to recurrent calcium oxalate stone disease. The intervention is to be viewed as a possible addition or research head, but not as a substitute to standard care. Further development should be justified by replication, safety, cost, standardization, acceptability and comparison of available alternatives.

In general, the example results confirm the study hypothesis and give a logical framework of a real investigation. All numerical findings, ethical principle, registration, disclosure and analysis must be substituted or checked with the actual study record prior to scientific submission.

## 5. CONCLUSION

Hydroalcoholic Tribulus terrestris fruit extract showed a positive difference in crystal aggregation inhibition over vehicle control and potassium citrate reference with complementary differences in nucleation turbidity, mean crystal size and calcium oxalate monohydrate proportion in this complete manuscript draft using dummy/example data. The trend was similar to complexation of crystal-forming ions, adsorption to crystal surfaces, and protection of tubular epithelial cells and it was proposed to have potential relevance since an assay-linked botanical standard could be used to develop adjunctive strategies to address recurrent calcium oxalate stone disease. The draft offers an organized foundation to protocol development, data-collection planning, and subsequent manuscript preparation; nevertheless, all numerical data should finally be substituted by ethically produced and separately validated data to be able to present the work as an original piece of research.

Declarations for Completion Before Submission

**Ethics approval:** Insert the actual institutional ethics committee name, approval number, approval date, and relevant consent or animal-welfare statement.

**Trial registration:** Insert the registry name and number for prospective clinical trials, where applicable.

**Funding:** Insert the verified funding source or state that no specific funding was received.

**Conflicts of interest:** Insert author-specific conflict-of-interest disclosures.

**Data availability:** Describe where the actual de-identified dataset and analysis code will be available.

**Author contributions:** Insert contribution statements using an accepted taxonomy such as CRediT.

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