

PHYTOCONSTITUENTS AND IN VITRO ANTI-UROLITHIATIC ACTIVITY OF VARIOUS HYDROPHILIC EXTRACTS OF *TERMINALIA CATAPPA* LEAVES ON CALCIUM OXALATE CRYSTAL

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ABSTRACT

Background: Medicinal plants play an important role in the alternative or complement therapy to manage of urinary stones at this time. *Terminalia* genus was proved anti-urolithiatic activity via in vitro inhibition of calcium oxalate formation. Among the samples, *Terminalia catappa* showed as a potential plants for this activity in India. Moreover, they were a common plant species in Vietnam and there was no research on this topic in our country. **Objectives:** To evaluate in vitro anti-urolithiatic activity of *Terminalia catappa* leaves in Vietnam, through inhibition of calcium oxalate formation. In addition, moisture value, preliminary screening of the chemical composition and determination of tanninoid content of aqueous extract also were conducted. **Materials and methods:** Moisture content of herbs was conducted according to guidelines of 5th Vietnam pharmacopoeia, appendix 9.6. Qualitation of the phytochemical constituents of aqueous extract with appropriate reagents to confirm the presence of natural compounds via chemical reactions. Determination of tanninoid content of aqueous extract was evaluated by using oxidation-reduction titration method (Lowenthal assay) and skin powder method in instructing of appendix 12.6 from 5th Vietnam pharmacopoeia. Inhibitory effect of calcium oxalate formation of three hydrophilic extracts (45% ethanol, 96% ethanol and aqueous extracts) was confirmed by nucleation assay with cystone as a positive control. **Results:** Moisture content of medicinal plant was 11.625%. *Terminalia catappa* leaves contain the major phytochemical constituents such as flavonoids, tanninoids and saponins. The tanninoid content according to Lowenthal method and skin powder method were 10.88% and 10.70%, respectively. Therefore, the average tannin content was confirmed to be approximately 10.79%. Among the investigated samples, aqueous extract showed the best inhibitory activity of calcium oxalate crystal formation with an IC_{50} of 602.67 $\mu\text{g/mL}$ when compared to cystone with IC_{50} 423.05 $\mu\text{g/mL}$. **Conclusions:** The aqueous extract from *Terminalia catappa* leaves has been shown to be a promising source for anti-calcium oxalate crystals formation activity on experimental model. The anti-urolithic potential of *Terminalia catappa* leaves may be related to its major phytoconstituents.

Keywords: *Terminalia catappa*, tanninoid, nucleation assay, calcium oxalate.

I. INTRODUCTION

Kidney stones (nephrolithiasis) were formed by the deposition of crystals in the kidney. Urinary stone (urolithiasis) was a condition that occurs when these stones escaped the kidney and moved into other part of the urinary system, including the ureters, bladder, and urethra. These stones have been associated with urinary tract obstruction, kidney failure, and urinary infections [1]. According to an epidemiological survey of urolithiasis in Asia, the prevalence rate was 5-19.1% in West Asia, Southeast Asia, South Asia and some developed countries (Korea and Japan). Meanwhile, this percentage was only 1-8% in most of East Asia and North Asia. In addition, calcium oxalate was the most common kidney

stone composition, accounting for 75-90%, followed by uric acid (5-20%), calcium phosphate (6-13%), struvite (2-15%), apatite (1%) and cystine (0.5-1%) [2]. In Vietnam, the estimated prevalence rate of urolithiasis ranged from 2-12%. According to a recent report, this percentage shows an upward trend according to the data from 1990 to 2019 [3].

Data from *in vitro*, *in vivo* and clinical trials suggested that herbs may be considered as an alternative or adjunct therapy in urolithiasis treatment [4]. In India, species of the genus *Terminalia* have been demonstrated to be a promising medicinal product for urolithiasis treatment through *in vitro* screening data [5]. Based on the above information, this study aims to evaluate the *in vitro* inhibitory effect against urinary stones on an anti-calcium oxalate crystal formation model and phytochemical data of *Terminalia catappa* leaves, a species of the genus *Terminalia* in Vietnam.

II. MATERIALS AND METHODS

2.1. Materials

Terminalia catappa leaves, collected in August 2021 in Ninh Kieu district, Can Tho city, Vietnam. Fresh leaves (3 kg) were thoroughly washed, damaged parts were removed. After that, the samples were left to dry, then later dehydrated at 60 °C until dry. The samples were then ground to a fine powder and used for further assay.

2.2. Methods

2.2.1. Preliminary phytochemical evaluation of *Terminalia catappa* leaves

Determination of the moisture

According to the instructions in Vietnamese Pharmacopoeia V, Appendix 9.6. [6]

Preliminary identification of phytochemical components

Qualitative analysis to identify alkaloids, flavonoids, anthocyanins, proanthocyanidins, tanninoids, saponins according to literature [7].

Tanninoid quantification

Oxidation-reduction titration (Lowenthal assay)

The experiment was conducted according to Wangiyana et al (2019) [8]. Take 5 mL of the sample solution into a conical flask, add 150 mL of distilled water, 5 mL of indigo carmine solution, and then quantify with KMnO_4 , drip and shake well until a yellow color appears, then stop. Simultaneously, quantify a blank sample with 5 mL of indigo carmine solution dissolved in 150 mL of water. 1 mL of KMnO_4 equivalent to 4,157 mg of tannins. The tanninoid content was calculated using the formula (2.1):

$$X = \frac{(a - b) \times V_2 \times 0,0004157 \times 100}{V_1 \times G} (\%)$$

X: tannin content, calculated as dry herbs (%); a: mean volume of KMnO_4 in sample titration (mL); b: mean volume of KMnO_4 in blank titration (mL); V_1 : volume of sample solution to be quantified (10 mL); V_2 : volume of the flask (250 mL); G: Weight of the herbs, minus moisture (g)

Skin powder method

The experiment was conducted according to Vietnam Pharmacopoeia V, Appendix 12.6 [6]. Three samples were weighed and recorded mass as T_1 , T_2 and T_0 (gram), respectively. The percentage (%) of tanninoids was calculated using the formula (2.2):

$$\frac{(T_1 - T_2 + T_0) \times 10 \times 100}{a}$$

In which, a represented the mass of the herbs, minus moisture (g)

2.2.2. *In vitro* anti-calcium oxalate crystals formation assay

Condensed extracts preparation: The 45% and 96% ethanol (EtOH) extracts were carried out by soaking technique. Samples of *Terminalia catappa* leaf powder (500 grams) were soaked with 45% EtOH and 96% EtOH solvents. After 24 hours, collected the extract and filtered it through filter paper. Repeated the procedure until the extraction was completed, collected the extract and recovered the solvent by rotavap, collected the corresponding condensed extract. The aqueous extract was performed by hot extraction technique. Herbal powder (500 grams) was heated with distilled water, repeated 3 times, the liquid extract and vacuum freeze-dry was collected, the corresponding condensed extract was collected. After that, the condensed extract content of all samples was calculated by percentage equation.

Nucleation assay: Carried out according to Abu et al (2020) [9], with appropriate adjustments. Accurately take 100 μ L of each test sample (45% EtOH extract, 96% EtOH extract, aqueous extract and positive control) at different concentrations into separate eppendorfs containing 950 μ L CaCl_2 . The crystal formation reaction started with the addition 950 μ L $\text{Na}_2\text{C}_2\text{O}_4$. The reaction mixture was incubated in the dark at 37 $^\circ\text{C}$ for 15 min, then optical density was measured at 620 nm wavelength. All experiments were performed 3 times. Cystone will be used as a positive control. The percentage of inhibition (%) was calculated according to the formula (2.3) as follows:

$$\% \text{inhibition} = [\text{OD}_{\text{blank sample}} - \text{OD}_{\text{test sample}}] / \text{OD}_{\text{blank sample}} \times 100.$$

Statistical analysis: Calculate the percentage of inhibition of each condensed extract of different concentrations. From there, determine IC_{50} (concentration at which the condensed extract inhibits 50% of calcium oxalate crystals formation) of each hydrophilic extract. In the same extracts, the OneWay-ANOVA test was used to evaluate the difference in inhibitory effects at different concentrations.

III. RESULTS

3.1. Preliminary analysis of phytochemicals of *Terminalia catappa* leaves

3.1.1. Moisture

The average moisture of the samples of herbs after triplicate tests was 11.625%. This result was lower than value defined in the Vietnamese Pharmacopoeia (13%). The moisture was suitable for storage and use.

3.1.2. Content of condensed extracts

The results of the content of 96% EtOH, 45% EtOH and aqueous extracts were presented in Table 1. The highest value belonged to 96% EtOH extract with 8.24%, followed by 45% EtOH extract with 6.01% and aqueous extract with 2.99%, respectively.

Table 1. Contents (%) of condensed extracts from *Terminalia catappa* leaves

Extracts	Mass (g)	Contents (%)
EtOH 96%	41.18	8.24
EtOH 45%	30.05	6.01
Aqueous	14.96	2.99

3.1.3. Preliminary qualitative analysis of phytochemical composition

In general, aqueous extract of *Terminalia catappa* leaves contains noticeable chemical components such as flavonoid, tanninoid and saponin (Table 2). These phytochemicals may be major components affecting the biological properties of *Terminalia catappa* leaves.

Table 2. Phytochemical composition of aqueous extract of *Terminalia catappa* leaves

Natural compounds	Reagents used	Results
Alkaloid	Wagner	-
	Dragendorff	-
	Valse- Mayer	-
Flavonoid	FeCl ₃ 5%	+
	Cyanidin	+
Anthocyanoside	HCl 10%, NaOH 10%	-
Proanthocyanidin	HCl 10%	-
Tanninoid	Gelatin	++
	FeCl ₃ 5%	++
Saponin	Shaked with water	++

3.1.4. Tanninoid content

Oxidation titration method (Lowenthal method)

The results of average volume values V (mL) used for titration of extracts and blank sample of 0.1N KMnO₄ were 15.26 mL and 1.13 mL, respectively. Using formula (2.1) to calculate, the tanninoid content was 10.88%.

Skin powder method

The mass results (g) of three test sample solutions after testing were presented in table 3. Apply formula (2.2) to calculate the tanninoid content was 10.70%.

Table 3. Mass (g) of 03 samples of test solution

Samples	Mass (g)
T ₀	0.128
T ₁	0.141
T ₂	0.162

Through 2 methods, the average tanninoid content was estimated about 10.79%.

3.2. Inhibition effect on *in vitro* calcium oxalate kidney stone formation

The results of inhibition of calcium oxalate kidney stone formation *in vitro* on different hydrophilic extracts from *Terminalia catappa* leaves were shown in Table 4.

Table 4. Inhibition effect on calcium oxalate kidney stone formation *in vitro*

Concentration (μg/mL)	OD values (% Inhibition effect)			
	Aqueous ex.	45% EtOH ex.	96% EtOH ex.	Cystone
100	0.0716 ± 0.0021a (18.26%)	0.0720 ± 0.0027a (17.86%)	0.0813 ± 0.0015a (7.21%)	0.0713 ± 0.0032a (18.63%)
200	0.0637 ± 0.0015b (27.37%)	0.0600 ± 0.0010b (31.55%)	0.0720 ± 0.0010b (17.83%)	0.0543 ± 0.0012b (38.02%)

Concentration ($\mu\text{g/mL}$)	OD values (% Inhibition effect)			
	Aqueous ex.	45% EtOH ex.	96% EtOH ex.	Cystone
400	$0.0580 \pm 0.0017\text{b}$ (33.83%)	$0.0526 \pm 0.0015\text{b}$ (39.92%)	$0.0667 \pm 0.0006\text{c}$ (23.95%)	$0.0420 \pm 0.0010\text{c}$ (52.09%)
600	$0.0437 \pm 0.0035\text{c}$ (50.20%)	$0.0460 \pm 0.0036\text{c}$ (47.53%)	$0.0590 \pm 0.0000\text{d}$ (32.69%)	$0.0343 \pm 0.0031\text{d}$ (60.85%)
800	$0.0363 \pm 0.0021\text{d}$ (58.55%)	$0.0413 \pm 0.0006\text{d}$ (52.84%)	$0.0503 \pm 0.0012\text{e}$ (42.58%)	$0.0233 \pm 0.0006\text{e}$ (73.39%)
1000	$0.0263 \pm 0.0015\text{e}$ (69.95%)	$0.0357 \pm 0.0024\text{e}$ (59.31%)	$0.0433 \pm 0.0006\text{f}$ (50.57%)	$0.0153 \pm 0.0021\text{f}$ (82.51%)

Values followed by different letters in the same column were statistically different at the 5% level.

Based on the percentage of inhibition effect, the regression equations were established and the IC_{50} values ($\mu\text{g/mL}$) were calculated and presented in Table 5.

Table 5. Regression equation and IC_{50} of test samples

Samples	Regression equation	IC_{50} ($\mu\text{g/mL}$)
Cystone	$y = 0.0007x + 0.2039$ ($R^2 = 0.9516$)	423.05
Aqueous extract	$y = 0.0006x + 0.1384$ ($R^2 = 0.9899$)	602.67
45% EtOH extract	$y = 0.0004x + 0.1984$ ($R^2 = 0.9362$)	754.02
96% EtOH extract	$y = 0.0005x + 0.0554$ ($R^2 = 0.9852$)	889.20

IV. DISCUSSION

4.1. Preliminary analysis of phytochemical compositions

4.1.1. Preliminary phytochemical compositions

In general, aqueous extract contains chemical components such as flavonoid, tanninoid and saponin. These data is similar to the qualitative analysis of *Terminalia catappa* leaves in São Paulo, Brazil and Hanoi, Vietnam according to Katiki *et. al.* (2017) [10] and H.D. Hung *et. al.* (2022) [11], respectively. The phytochemical compositions will certainly influence the biological effects of herbal medicine.

4.1.2. Content of tanninoid

The result showed the contents of tanninoid in both quantitative methods were similar. The average content was estimated about 10.8%. This result was approximately 10 times higher than that in the research of Lokman *et. al.* (2014) with total tanninoid content of *Terminalia catappa* leaves harvested in Malaysia was 1.15% [12]. However, the used solvent in this article was 70% acetone. Tanninoids were polar compounds, thus water was selected for extraction in our study. Besides, another research done in Indonesia showed that the tanninoid content in *Terminalia catappa* leaves was 12.45% with the solvent being 85% ethanol [13]. Hereby, it is important to select an appropriate solvent to take the highest active ingredient content. Besides, water is a friendly solvent, and easy to be used in practice compared to acetone or ethanol.

4.2. Inhibition of calcium oxalate crystallization *in vitro*

The three hydrophilic extracts of *Terminalia catappa* leaves show the inhibitory activity for crystallization in the concentration range being 1000-100 $\mu\text{g/mL}$. In which, the

aqueous extract showed the best inhibitory activity. If its concentration was increased from 100 to 1000 µg/mL, the OD value gradually decreased from 0.0716 to 0.0263, accordingly, the inhibitory effect for crystallization increased from 18.26% to 69.95%. Continuously, 45% EtOH extract showed the inhibition results was from 17.86-59.31%. Finally, 96% EtOH extract showed the lowest inhibitory activity, and its inhibitory effect was only from 7.11%-50.57%. Besides, it can be seen that for the aqueous extract and 45% EtOH extract, the inhibitory effects for calcium oxalate crystallization were not significantly different with the two concentration levels being 200 µg/mL and 400 µg/mL. The IC₅₀ values of aqueous extract, 45% EtOH extract and 96% EtOH extract were 602.67 µg/mL, 754 µg/mL and 889.20 µg/mL, respectively, in comparison with IC₅₀ of cystone as a positive control which was 423 µg/mL. The results represented that the aqueous extract of *T. catappa* leaves was the most potential inhibitory effect in experimental model.

The data showing the similarity with the previous research about urinary stone inhibition *in vitro* on species of the genus *Terminalia* in India, in which *Terminalia cappata* showed its potentiality in the assays. The authors assumed that natural products in herbal medicine can prevent urinary supersaturation and crystal growth inhibition [5]. The anti-urolithic properties of *T. catappa* leaves may be indeed contributed by the chemical components. Based on the qualitative outcomes, the aqueous extract of *T. catappa* leaves contains the main compounds such as saponin, flavonoid and tanninoid. The data showed the saponin was effective at inhibiting the formation of urinary stone through the previous literatures on extracts of *Daucus carota*, *Kalanchoe pinnata*, and *Bergenia ciliata* [14]. Besides, the inhibitory activity for calcium oxalate stone formation of phlorotannin-rich extract from *Sarghassum wightii* was also proved. On that basis, the tanninoid may play a significant role in this activity of plants, while the content of tanninoid in the aqueous extract of *T. catappa* leaves was very high [15]. In addition, natural flavonoids have been demonstrated to effectively inhibit calcium oxalate stone formation both *in vitro* and *in vivo* in many researches. Therefore, flavonoids in *T. catappa* appeared to be an important component in preventing crystal formation [16].

V. CONCLUSION

Terminalia catappa leaves contain important phytochemical constituents such as flavonoid, tanninoid and saponin. The content of tanninoid studied in the redox titration method was 10.88% and the skin powder method was 10.70%, the average tannin content was 10.79%. The aqueous extract from herbal medicine of *Terminalia catappa* leaves showed the potential effect on inhibitory activity for calcium oxalate stone foundation *in vitro* with IC₅₀ was 602.67 µg/mL in comparison with cystone as a positive control being 423.05 µg/ mL.

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