

(RESEARCH ARTICLE)



## Assessment of antiurolithiatic activity of some herbal-fractions using *in vitro* techniques

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### Abstract

There is an extended inventory of medicinal plants used for urolithiasis due to their fewer side effects and because they contain copious phytochemicals that show advantageous effects in renal stones. The current research focuses on the *in vitro* evaluation of *Trigonella foenum-graecum* L., and *Nigella sativa* L., traditionally used in the treatment of urolithiasis by turbidity and titrimetric assay. Three different concentrations (1.0 mg/ml, 2.0 mg/ml, and 5.0 mg/ml) of the plant extracts were tested in each assay. The methanolic seed extracts of *Trigonella foenum-graecum* and *Nigella sativa* at 5.0 mg/ml exhibited a significant percentage of inhibition of calcium oxalate formation by turbidity method as well as percentage dissolution of calcium oxalate crystals by titrimetric method. The results of test groups displayed a near match to the standard drug Neeri used at 0.1mg/ml. This study has been primary evidence for *Trigonella foenum-graecum* and *Nigella sativa* as potential contributors to anti-urolithiasis.

**Keywords:** Renal stones; *Trigonella foenum-graecum* L.; *Nigella sativa* L.; Turbidity; Titrimetric assay

### 1. Introduction

Urolithiasis is a leading health problem that affects 12% of the global population [1]. The disorder results from the synergistic effect of biochemical, epidemiological, and genetic factors. It is estimated that one in ten people will have a kidney stone at some time in their lives. The risk of kidney stones is about 11% in men and 9% in women [2]. Other diseases such as high blood pressure, diabetes, and obesity may add to the risk of kidney stones [3,4].

Kidney stones are hard aggregations of minerals and acid salts that can be painful while passing through the urinary tract. Among several types of renal calculi, calcium-containing stones are the most common type of calculi representing 80% of urinary calculi [5]. The calcium-containing stones may occur in the form of calcium oxalate, calcium phosphate, or a combination of both. Magnesium phosphate, cysteine, and uric acid to some extent also contribute to the formation of renal stones [6]. Kidney stone genesis involves multiple phytochemical steps with the inception of crystal nucleation, aggregation, and finally retention in the urinary system [7].

The current management of urolithiasis through Extracorporeal Shock Wave Lithotripsy (ESWL), ureteroscopy (URS), and percutaneous nephrolithotomy (PNL) or by consuming synthetic drugs have multiple side effects and are quite costly [8]. Medicinal plants have been used traditionally to treat kidney stones even before the invention of modern treatments as it is proven to be effective and naturally safe remedies. Thus, treatment with the medicinal plant is recommended as the range of phytochemicals they contain may be associated with anti-urolithiasis properties [9]. There are sufficient research statistics to support the use of herbal medicine as an alternate therapy in the treatment of urolithiasis. *In vitro* studies confirmed the significant anti-urolithiasis activity of *Achyranthes aspera* [10], *Semecarpus anacardium* [11], *Terminalia chebula* [11], *Tinospora cordifolia* [11], *Paronychia argentea* [12] and *Tribulus terrestris*

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[13]. *Cuminum cyminum* essential oil has displayed satisfactory results by reducing the growth of calcium oxalate crystals and by inhibiting the formation of renal stones in an *in vivo* study on mice [14].

Fenugreek (*Trigonella foenum-graecum*), a fragrant herb of the pea family Fabaceae, and *Nigella sativa*, commonly known as kalonji or black seeds, of the family Ranunculaceae, have a long history of medicinal use. Both herbs are loaded with copious amounts of phytoconstituents that comprise alkaloids, phenols, terpenes, fatty acids esters, and saponins [15,16,17,18]. Trigonelline and diosgenin, the major constituents of fenugreek seeds have been documented to possess various pharmacological activities [19]. An *in vivo* study has confirmed the protective effects of fenugreek against pesticide-induced damage to the kidney and liver [20]. In another *in vivo* study fenugreek has proved a potent antioxidant and anti-urolithiatic candidate against ethylene glycol-induced kidney calculi in rats [21]. Owing to its strong biological activities such as hepatoprotective, antimicrobial, anti-diabetic, anti-inflammatory, antifertility, and cytotoxic activity, *Nigella sativa* is advocated as a miracle medicinal herb [22]. Investigations revealed thymoquinone, a major phytoconstituent of kalonji seeds, with anti-inflammatory, antioxidant, and immunomodulatory effects when administered at a dose of 5 mg/kg to ethylene glycol-induced calcium oxalate kidney calculi in rats, significantly decreased the number and size of calcium oxalate crystals [23,24]. It has been reported that *Nigella sativa* seeds mixed with honey induced the breakdown of renal calculi and subsequent excretion from the body [25].

The current study aims to evaluate the anti-urolithiasis activity of *Trigonella foenum-graecum* and *Nigella sativa* seed extract to collate empirical data that will reinforce the existing anti-urolithiasis considerations and could be used as a pilot source for the formulation of therapeutics.

## 2. Materials and Methods

### 2.1. Collection and processing of plant materials

The plant materials were collected from the local market. Seeds of *Trigonella foenum-graecum* and *Nigella sativa* were directly powdered. The powdered samples were stored in a clean airtight container until further use.

### 2.2. Preparation of extracts

Extraction was done by the Soxhlet apparatus using methanol as a solvent. 10 g of each dried powder sample was weighed and placed in a thimble chamber. The extraction process was carried out by using 100 ml of methanol for 48 hrs. followed by drying in a rotary vacuum evaporator. The dried sample extracts were stored in a refrigerator for further use.

### 2.3. In-vitro evaluation of anti-urolithiasis activity

#### 2.3.1. Turbidity method (Nucleation assay)

**Table 1** Preparation of various samples for anti-urolithiasis activity by turbidity assay

Sample	CaCl <sub>2</sub> (ml) 50mM in Tris buffer pH 6.5	NaO 75mM in Tris buffer pH 6.5	Distilled Water(ml)	Seed extract (ml)			Standard (Neeri) (ml)
				1.0 mg/ml	2.0 mg/ml	5.0 mg/ml	
Test tube 1 (Negative control)	0.95 ml	0.95 ml	1				0.1 mg/ml
Test tube 2	0.95 ml	0.95 ml		1			
Test tube 3	0.95 ml	0.95 ml			1		
Test tube 4	0.95 ml	0.95 ml				1	
Test tube 5 (Positive control)	0.95 ml	0.95 ml					1

The *in vitro* anti-urolithiasis activity was tested in terms of inhibition of calcium oxalate (CaOx) formation in the presence of the standard drug, fenugreek, and kalonji seed extracts following the method of Khare et al with slight modification [26]. Turbidity in each assay was measured at 620nm using a UV/Vis spectrophotometer. To study the effect of inhibitors three different concentrations (1.0 mg/ml, 2.0 mg/ml, and 5.0 mg/ml) each of MSETFG (Methanolic seed extract of *Trigonella foenum-graecum*) and MSENS (Methanolic seed extract of *Nigella sativa*) and the standard drug at 0.1 mg/ml were prepared and assayed as depicted in Table 1.

The turbidity resulted immediately by mixing the solutions of all experimental sets (control, test, and standard) was measured using a UV/Vis spectrophotometer at 620nm. The absorption was carried out at an interval of 1 min. for 5 min. (Table 2).

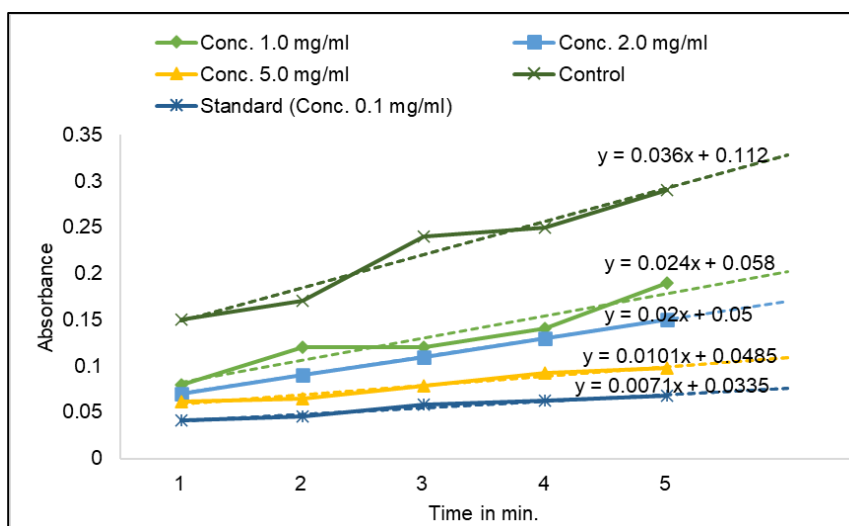
**Table 2** The absorbance of different samples at 620 nm in turbidity assay

Sample	Conc. mg/ml	OD after 1 min.	OD after 2 min	OD after 3 min	OD after 4 min	OD after 5 min
Control		0.15	0.17	0.24	0.25	0.29
MSETFG	1.0	0.08	0.12	0.12	0.14	0.19
	2.0	0.07	0.09	0.11	0.13	0.15
	5.0	0.061	0.065	0.078	0.092	0.098
MSENS	1.0	0.10	0.12	0.15	0.18	0.21
	2.0	0.062	0.063	0.079	0.091	0.095
	5.0	0.07	0.09	0.11	0.15	0.15
Standard (Neeri)	0.1	0.041	0.045	0.058	0.062	0.068

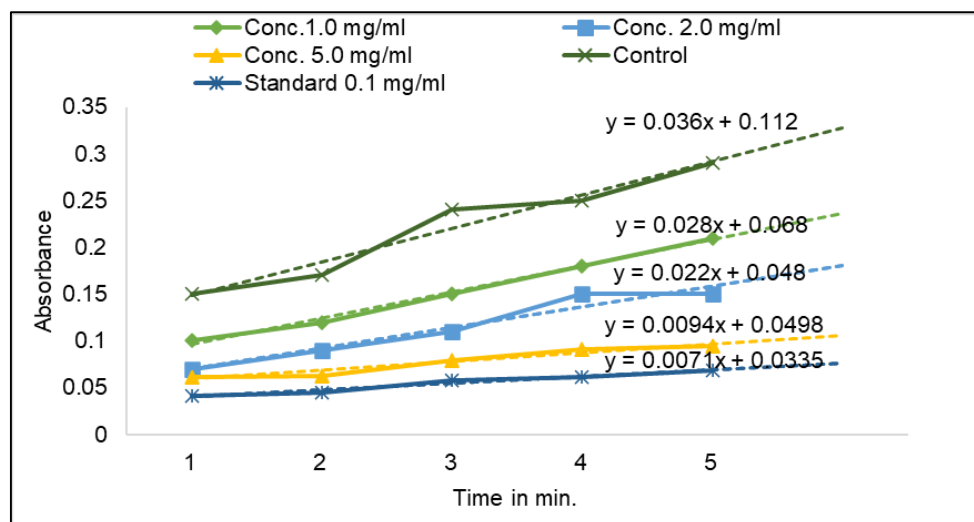
The regression equation for each graph was calculated which was used to determine the slope of the graph (Fig. 1 & 2). The percentage inhibition of nucleation was calculated using a graphical method by employing the following formula:

$$\text{Inhibition (\%)} = [1 - (S_i / S_c)] \times 100$$

(Where  $S_i$  is the slope of the graph in the presence of inhibitors (drugs/seed extracts); and  $S_c$  is the slope of the graph without inhibitors (negative control).



**Figure 1** Change in turbidity in the absence and presence of *Trigonella foenum graecum* L. seed extract and standard at 620nm (The equation displayed with each graph is the regression equation)



**Figure 2** Change in turbidity in the absence and presence of *Nigella sativa* L. seed extract and standard at 620nm (The equation displayed with each graph is the regression equation)

### 2.3.2. Titrimetric method (Calcium oxalate dissolution assay)

This method was used to evaluate the activity of the plant extracts in dissolving the already-formed stones in the kidney [27].

#### Preparation of calcium oxalate crystals

1.47 gm of calcium chloride dihydrate was dissolved in 100 ml of distilled water and 1.34 gm of sodium oxalate was dissolved in 100 ml of 2N H<sub>2</sub>SO<sub>4</sub>. Both solutions were mixed equally in a beaker to precipitate out calcium oxalate with constant stirring. The resulting precipitate of calcium oxalate was freed from traces of H<sub>2</sub>SO<sub>4</sub> by washing it with an ammonia solution followed by washing it with distilled water and dried at a temperature of 60°C (Fig. 3).

#### Preparation of the semi-permeable membrane from farm eggs

The semipermeable membrane of eggs lies in between the outer calcified shell and the inner contents like albumin and yolk. The apex of the eggs was pierced by a glass rod to squeeze out the whole content. Empty egg membranes were washed thoroughly with distilled water and placed in a beaker consisting of 2N HCl overnight, which caused complete decalcification. Then, the semi-permeable membranes were washed with distilled water, placed in an ammonia solution for neutralization of acid traces, and rinsed with distilled water. Finally, the egg membranes were stored in 2% ammonia until used (Fig. 4).



**Figure 3** Calcium oxalate crystals



**Figure 4** Semi-permeable membranes prepared from farm eggs

#### Estimation of calcium oxalate (CaOx) by titrimetry

Precisely 5 ml of 1mg/ml of calcium oxalate with 2 ml of 1.0 mg, 2.0 mg, and 5.0 mg/ml of each plant extract were packed separately in the semi-permeable membranes of the egg. These packings were allowed to suspend in a conical

Erlenmeyer flask containing 100 ml of 0.1M Tris [Tris (hydroxymethyl) aminomethane] buffer. All the conical flasks were placed in a water bath at 37 °C overnight. Then, the semi-permeable membranes along with the inner contents were removed from the conical flasks. 2 ml of 1N H<sub>2</sub>SO<sub>4</sub> was added to the contents and was titrated with 0.1N of KMnO<sub>4</sub> till a light pink color was obtained. One group containing 5 ml of calcium oxalate with 2 ml distilled water served as negative control. The second group containing 5 ml of calcium oxalate and 2 ml of 0.1 mg/ ml standard drug served as a positive control. The 3rd, 4th, and 5th groups contained 5 ml of calcium oxalate and 2 ml of methanolic extracts at a concentration of 1.0, 2.0, and 5.0 mg/ml of each plant sample were used as test groups.

All the assessments were performed in triplicate and the amount of calcium oxalate dissolved was calculated using the mole concept as follows:

$$\text{Dissolution (\%)} = [(C - T)/C] \times 100$$

(Where C is the precipitate of calcium oxalate remained in control; and T is the precipitate of calcium oxalate remained when test solution/standard is used. Results were expressed as % dissolution of calcium oxalate  $\pm$  standard error.

(5 ml of 0.1 N KMnO<sub>4</sub> is equal to 1.898 mg of calcium)

### 3. Results and Discussion

#### 3.1. In-vitro evaluation of anti-urolithiasis activity by turbidity method

The percentage inhibition of nucleation calculated using the graphical method as stated earlier showed that methanolic seed extracts of *Trigonella foenum-graecum* and *Nigella sativa* respectively inhibited crystal formation as well as promoted crystal dissolution in a dose-dependent manner and were comparable with the activity of the standard drug Neeri. Both the plant extracts MSETFG and MSENS, taken as a sample showed their ability to inhibit CaOx formation in the range from 22.23 to 73.88 % (Table 3). MSENS was found to be more effective in inhibiting the CaOx crystal aggregation. These findings were consistent with the preventive activity of *Maerua angolensis* leaf extract [28] and *Argania spinosa* fruit and *Acacia senegal* gum powder extract on calcium oxalate crystallization [29].

**Table 3** Percentage inhibition of calcium oxalate formation evaluated using turbidity method

Sample	Conc. (mg/ml)	Regression equation	Slope	% Inhibition
Control	—	$y = 0.036x + 0.112$	0.036	—
MSETFG	1.0	$y = 0.024x + 0.058$	0.024	33.34
	2.0	$y = 0.02x + 0.05$	0.02	44.45
	5.0	$y = 0.0101x + 0.0485$	0.010	72.23
MSSENS	1.0	$y = 0.028x + 0.068$	0.028	22.23
	2.0	$y = 0.022x + 0.048$	0.022	38.89
	5.0	$y = 0.0094x + 0.0498$	0.0094	73.88
Standard	0.1	$y = 0.0071x + 0.0335$	0.0071	80.28

The values represent mean $\pm$ SD

#### 3.2. In vitro evaluation of anti-urolithiasis activity by titrimetry method

This technique evaluates the anti-urolithiasis activity by dissolving the artificial CaOx packed in a semipermeable membrane of an egg with different plant samples. The amount of CaOx dissolved was used as the indicator to evaluate anti-urolithiasis activity. A considerable percentage of dissolution activity, 60 $\pm$ 0.96 and 75 $\pm$ 2.34 by MSETFG and MSENS was reported at 5.0 mg/ml concentration respectively (Table 4).

**Table 4** Percentage dissolution of calcium oxalate crystals using titrimetry method

Sample	Conc. (mg/ml)	% Dissolution
MSETFG	1.0	47±1.34
	2.0	51±1.89
	5.0	60±0.96
MSENS	1.0	52±2.10
	2.0	60±1.56
	5.0	75±2.34
Standard (Neeri)	0.1	92±1.75

The values represent the % dissolution mean±SD

The observed anti-urolithiasis activity of *Trigonella foenum-graecum* and *Nigella sativa* methanolic seed extracts can be attributed to the array of phytoconstituents they contain. A study has revealed the effectiveness of fenugreek seed extract rich with trigonelline against renal crystal deposition in ethylene glycol-induced nephrolithiatic rats [30]. Sufficient research data supports the presence of phenolics, flavonoids, and isoflavones in fenugreek seed extract with antioxidant and anti-inflammatory properties [31,32]. In addition, coumarins, anthracene derivatives, and glycosides have also been reported by HPTLC (High-Performance Thin Layer Chromatography) analysis [33]. An *in vitro* investigation on *Nigella sativa* as an inhibitor of calcium oxalate crystallization evaluated by turbidity methods exhibited 86.0% nucleation percentage inhibition and inhibition of crystal aggregation was recorded by 66.6% [34]. The beneficial effects of kalonji seeds could be attributed to phytoconstituents such as rutin, quercine, and kaempferol which show potent molecular docking activity against enzymes involved in urolithiatic activity [35]. The exact mechanism of anti-urolithiasis action of these bioactives is still undiscovered but it is reported that they may act through multiple channels by inhibiting the process of crystallization, nucleation, and aggregation, helping in the impulsive passage of calculi, regulating the urine output, pH, etc [36,37].

#### 4. Conclusion

The findings of the present study highlight the ability of tested plant samples to prevent the nucleation and dissolution of calcium oxalate crystals as proved by *in vitro* studies. *Trigonella foenum-graecum* and *Nigella sativa* have been used in ethnomedical practice in the treatment of urinary problems and renal stones from ancient times. The current investigation suggests the presence of anti-urolithic components in both herbs that require pre-clinical and clinical evaluation. This is a first-hand report of the evaluation of antiurolithiatic activity of fenugreek and kalonji seed extract by titrimetry method. This study has given a piece of basic scientific evidence to support the existing statistics related to anti-urolithiasis activity of *Trigonella foenum-graecum* and *Nigella sativa* and for the potential use of these herbs for a novel herbal drug formulation with anti-urolithiasis activity.

#### Compliance with ethical standards

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##### Disclosure of conflict of interest

The authors have no conflict of interest to disclose.

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