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ANTI- UROLITHIATIC ACTIVITY OF AQUEOUS EXTRACT OF *CLITORIA TERNATEA* LINN AGAINST ETHYLENE GLYCOL INDUCED UROLITHIASIS IN EXPERIMENTAL RODENTS

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ABSTRACT

Clitoria ternatea Linn (butterfly pea, Fabaceae), *Clitoria ternatea* is used to treat a variety of ailments and symptoms. It possesses antidepressant, anticonvulsant, anticancer, hypolipidemic, anti-inflammatory, analgesic and antipyretic qualities, as well as local anesthetic, purgative, and antidiabetic effects. It's also used to treat snake bites and scorpion stings in India. The leaves aqueous extract of *Clitoria ternatea* was investigated for its antiurolithiatic and diuretic activity. Ethylene glycol (0.75% in water) feeding resulted in hyperoxaluria as well as increased renal excretion of calcium and phosphate. Aqueous extract of *Clitoria ternatea* was given orally in curative and preventive regimens over a period of 28 days. Supplementation with extract significantly ($P < 0.001$) lowered the urinary excretion and kidney retention levels of oxalate, calcium and phosphate. Furthermore, high serum levels of urea nitrogen, creatinine and uric acid were significantly ($P < 0.001$) reduced by the extract. The results were comparable with the standard drug, cystone (750 mg/kg). The reduction of stone-forming constituents in urine and their decreased kidney retention reduces the solubility product of crystallizing salts such as calcium oxalate and calcium phosphate, which could contribute to the antiurolithiatic property of the extract. The extract exhibited significant diuretic activity at dose of 250 and 500 mg/kg body weight as evidenced by increased total urine volume and the urine concentration of Na^+, and K^+. These findings affirm assertions made regarding the effectiveness of the extract of this plant against urinary pathologies in the Indian folk medicine.

KEYWORDS: *Clitoria ternatea* Linn, Antiurolithiatic, Diuretic activity, Ethylene glycol, Cystone.

INTRODUCTION

Nephrolithiasis or renal stone disease remains a significant health problem in the adult population, with serious medical consequences, throughout a patient's lifetime. The worldwide incidence of urolithiasis is quite high, and more than 80% of urinary calculi are calcium oxalate stones alone or calcium oxalate mixed with calcium phosphate.[1] The present-day medical management of nephrolithiasis is either costly or not without side-effects. Invasive procedures for the treatment of nephrolithiasis may cause serious complications and also impose a great load of costs on the healthcare system. In contrast, traditional medicines have offered a substitute for many diseases and also have provided some supplementary information about the pathogenesis of diseases.[2] *Clitoria ternatea* Linn (CT) a perennial twing herb, steams are terete, more or less pubescent belongs to the family Fabaceae. There are two varieties of Clitoria ternatea white-flower and blue flower varieties. *Clitoria ternatea* locally known as shankhpushpi. Shankhpushpi is one of the medhyarasayana plant and is reported to promote intellectual capacity, rejuvenate the body and nervous tissue, enhance the aura of the body and improve general health to elicit quality ageing. The roots, seeds and leaves of *Clitoria ternatea* have long been widely used as a brain tonic and is believed to endorse memory and intelligence. It is reported to have antidepressant, anticonvulsant, antiinflammatory, analgesic and antipyretic, local anesthetic, purgative and anti-diabetic activity. It is also used for treatment of snakebite and scorpion sting in India [3-9]. The objective of the present study was to investigate and to validate the antiurolithiatic property of *Clitoria ternatea* extract in experimentally-induced urolithiasis in rats.

MATERIALS AND METHODS

Collection and identification of plant material

The leaves of *Clitoria ternatea* was collected locally from Bhopal, M.P. After cleaning, plant parts were dried under shade at room temperature for 3 days and then in oven at 45°C till complete dryness. Dried plant parts were stored in air tight glass containers in dry and cool place to avoid contamination and deterioration. The medicinal plant *Clitoria ternatea* was authenticated by a plant taxonomist in order to confirm its identity and purity.

Chemical reagents

All the chemicals used in this study were obtained from Hi Media Laboratories Pvt. Ltd. (Mumbai, India), Sigma Aldrich Chemical Co. (Milwaukee, WI, USA), SD Fine-Chem Chem. Ltd. (Mumbai, India) and SRL Pvt. Ltd. (Mumbai, India).All the chemicals used in this study were of analytical grade.

Extraction of plant material

Powdered leaves of *Clitoria ternatea* 1500gm was kept for maceration with 2000ml of water for 24hours during successive extraction. The extract was double filtered using muslin cloth and Whatman filter paper No: 1and the extract concentrated and dried on water bath. The different concentration of aqueous extract was prepared for further study.[10]

Evaluation of antilithiatic activity

Experimental animals

Male albino wistar rats weighing between 150-200gm were used. The animals were fed with commercial rat feed pellets (Amrut laboratory animal feed Ltd. Sangli, India) and were given water ad *libitum.* They were housed in polypropylene cages under proper humidity conditions (temperature: 25 ± 2°C) and maintained on normal 12-12 h day-night cycle. The experimental protocol and all the procedures were approved by Institutional animal ethical committee.

Ethylene glycol induced urolithiasis model

While there are several animal models that are used to study hyperoxaluria and its consequences, the most commonly employed and simplest approach to induce hyperoxaluria is to provide ethylene glycol (EG) in an animal's drinking water.[11] Ethylene glycol (0.75%v/v) induced hyperoxaluria model was used to assess the antilithiatic activity in albino rat. Ethylene glycol was prepared in water (0.75% v/v) and stored in bottles before commencement of treatment. Ethylene glycol is reported to be renotoxic, urine parameters were estimated at the end of study to assess kidney functions. Ethylene glycol major toxicities are as a result of it being metabolized to oxic metabolite by an enzyme alcohol dehydrogenase.[12] Ethylene glycol is readily absorbed along the intestine and is metabolized in the liver to oxalate. When ethylene glycol is metabolized by the body it produces four toxic metabolites, they are glycoldehyde, glycolate, glycolic acid and glycoxalate. These metabolite causes tissue destruction primarily from calcium oxalate crystal deposition. Oxalic acid combines with calcium to form calcium oxalate crystals, which deposits in the kidney.

Experimental design

Animals were divided into five groups containing three animals in each group. Lithiasis was induced by the administration of 0.75% ethylene glycolated water to all groups viz., Lithiatic, standard and test groups except the normal control for 28 days. The lithiatic group were not received any drug. The standard group was received antiurothiatic drug cystone (750 mg/kg) from 15th day and test groups were maintained in two regimens as curative and preventive. In curative regimen the aqueous extracts of leaves of *Clitoria ternatea* was administered from 15th day and in preventive regimen the aqueous extract of *Clitoria ternatea* was given from lsl day onwards. All groups were maintaining on commercial pellet diet for 28 day.

Assessment of antiurolithiatic activity

Collection and analysis of urine

On 14th and 28th day all animals were kept in individual metabolic cages and urine samples were collected for 24hrs in measuring cylinder. During urine collection the animals were free access of normal water but not food. The collected urine sample was analyzed for calcium, magnesium, oxalate, creatinine and phosphate using standard methods. The urinary volume of all groups was also noted.[13] Calcium and magnesium were determined by colorimetric method; oxalate was determined by Hodgkinson and William's method, Phosphate using Fiske and subbarow method.

Microscopic examination of urine

For microscopy, 1 ml of the fresh urine sample was centrifuged at 3,000 rpm (revolutions per minute) for 10 minutes, and then 950pl of the supernatant was discarded. The crystals were identified by light electron microscope.

Serum analysis

After the experimental period, animals will be anaesthetized with diethyl ether. Blood was collected from the tail vein in non heparinized tubes and centrifuged at 2000 rpm for 20 min to obtain serum and is analysed for creatinine, BUN (blood urea nitrogen), and uric acid.[14]

Evaluation of diuretic activity

Experimental design

Male albino wistar rats weighing between 150-200gm were used. The animals were grouped into four of three animals each and they were fasted and deprived of food and water for 18 hours prior to the experiment. The first group received only 0.9% Nacl solution 25 ml/kg, p.o. The second group served as the standard group, received the standard drug furosemide 20 mg/kg ,p.o. Rest of the two groups received aqueous extract of leaves of *Clitoria*

ternatea of 250 mg/kg and 500 mg/kg suspended in 0.9% Nacl solution. All the animals received priming dose of 0.9% Nacl solution (25 ml/kg, p.o).[15]

Group I Normal Control	0.9% sodium chloride solution 25ml/kg, p.o.
Group II Standard	0.9% sodium chloride solution 25 ml/kg, p.o.+ standard drugFurosemide 20 mg/kg,p.o.
Group III Dose I	0.9% sodium chloride solution 25 ml/kg,p.o+ Aqueous extract of *Clitoria ternatea* (250 mg/kg bodyweight, p.o)
Group IV Dose II	0.9% sodium chloride solution 25 ml/kg,p.o+ Aqueous extract of *Clitoria ternatea* (500mg/kg bodyweight, p.o).

Assessment of diuretic activity

Collection and analysis of urine

After oral administration each animal were placed in an individual metabolic cages specially designed to separate feces and urine at room temperature. The observed parameters were total urine volume for 5 hours. Na+ K+ and CI- excreted in urine. The concentration of the electrolytes in urine is expressed in terms of mmol/1 and the urine volume is expressed in ml/100g/5 hours. Na+, K+ concentrations were measured by Flame photometer and Cl- concentration was estimated by titration with silver nitrate solution (N/50) using three drops of 5% potassium chromate as an indicator.[15]

Statistical analysis

The results were expressed as mean ± S.D. Difference among data were determined using one way ANOVA (software) followed by Dunnetts test as per suitability $P < 0.05$ was considered as significant, $P < 0.01$ was considered as very significant.

RESULTS

Urinary analysis

In the present study, chronic administration of 0.75% (v/v) ethylene glycolated water to male albino rats resulted in hyperoxaluria. On 14th day, the concentration of oxalate, and phosphate were increased in lithiatic, standard, and curative regimen when compared to normal. However, the treatment with aqueous extract of *Clitoria ternatea* in preventive regimen reduced elevated levels of these ions significantly ($p < 0.01$) when compared with lithiatic control group. Contradictorily, the excretion of magnesium and calcium levels were reduced in lithiatic, standard and curative regimen but these magnesium level were significantly increased ($p < 0.01$) in preventive regimen when compared with lithiatic control group, because the standard and curative regimen doses has not received any treatment up to 14th day Table 1.The concentrations of the various ions in the collected urine were investigated and found to fluctuate drastically after the treatment. On 28th day, the supplementation with standard (Cystone 750mg/kg) and curative and preventive regimen groups (aqueous extract *Clitoria ternatea* 500mg/kg) lowered the elevated levels of calcium, oxalate and phosphate when compared to lithiatic control group. In both curative and preventive regimens of aqueous extract of *Clitoria ternatea* treatment significantly decreased excretion ($p<0.01$) of calcium, oxalate, creatinine and phosphate when compared to lithiatic control group, while magnesium, one of stone inhibitor increased significantly ($p<0.0l$) than in lithiatic control Table 2.

Urine microscopy

The microscopic examination (200X) of urine of calculi induced rats (Group II) showed abundant, large crystals of Calcium oxalate with characteristic rectangular shape (Fig.1 A). The Standard drug Cystone treated animals showed very less or almost dissolved small crystals (Fig. 1B). On curative treatment, the aqueous extract of *Clitoria ternatea* showed better dissolution of the preformed crystals of Calcium oxalate (Fig. 1C); while, on preventive treatment, the Aqueous extract of *Clitoria ternatea* showed better prevention of stone formation along with the dissolution of preformed stones (Fig. 1D).

Serum analysis

The Serum Uric acid and blood urea nitrogen were markedly increased in calculi-induced animals while serum creatinine was slightly elevated in Group II indicating marked renal damage. However, aqueous extract of *Clitoria ternatea* treatment in curative (Group IV) and preventive (Group V) Regimen significantly ($p<0.01$) lowered the elevated serum levels of creatinine, uric acid and BUN (Blood Urea Nitrogen) Table 3.

Diuretic activity

Urine volume (ml), urine pH, concentration of Na+, K+, and CI- electrolytes (mmol/1) in the urine were recorded. The diuretic index of 5 hr urine samples was calculated to assess the diuretic potential of aqueous extract of *Clitoria ternatea*. The urinary volume of the standard (Furosemide) and aqueous extract of *Clitoria ternatea* (250 mg/kg and 500mg/kg) significantly increased ($p<0.01$) when compared to control group. The diuretic index (Lipschitz value) of standard drug was 5.14, the AECT 250mg/kg treatment shown 1.27 and the AECT 500mg/kg treatment shown. The sodium, potassium and chloride excretion of AECT treatment at both doses (250mg/kg and 500mg/kg) significantly ($p<0.01$) increased when compared to the control group. The standard drug also increased these ionic excretion levels significantly Table 4.

Table 1: Effect of aqueous extract of *clitoria ternatea* in urinary excretion of ions on 14th day.

Group	Water intake (ml/24h)	Urinary Volume (ml/24h)	Urinary pH	Urinary Excretion (mg/dl)			
				Calcium	Magnesium	Oxalate	Phosphate
G1. (Normal Control)	7.70 ± 0.09	7.67 ± 0.105	6.40 ± 0.06	4.14 ± 0.37	0.94 ± 0.018	0.34 ± 0.027	5.85 ± 0.42
G2. (Lithiatic Control)	15.28 ± 0.33	14.96 ± 0.23	5.82 ± 0.12	3.24 ± 0.07	0.75 ± 0.04	2.82 ± 0.11	8.54 ± 0.50
G3. (Standard Treated)	15.01 ± 0.14	14.82 ± 0.17	5.77 ± 0.07	3.21 ± 0.08	0.75 ± 0.06	2.96± 0.13	8.38 ± 0.33
G4. (Curative Treatment)	15.24 ± 0.28	14.86 ± 0.13	5.79 ± 0.07	3.26 ± 0.15	0.69 ± 0.06	2.72 ± 0.08	8.23 ± 0.27
G5. (Preventive Treatment)	9.36 ± 0.19*	9.12 ± 0.06*	6.63 ± 0.06*	4.62 ± 0.07*	1.95 ± 0.02*	1.23 ± 0.12*	6.24 ± 0.26*

Each value expressed as the mean ± SD (n=3) one- way ANOVA; *-P<0.01 when compared with lithiatic control group.

Table 2: Effect of aqueous extract of *clitoria ternatea* urinary excretion of ions on 28th day.

Group	Water intake (ml/24h)	Urinary Volume (ml/24h)	Urinary pH	Urinary Excretion (mg/dl)			
				Calcium	Magnesium	Oxalate	Phosphate
G1. (Normal Control)	7.72 ± 0.10	7.89 ± 0.12	6.42 ± 0.03	4.40 ± 0.59	0.96 ± 0.59	0.34 ± 0.013	5.83 ± 0.39
G2. (Lithiatic Control)	15.62 ± 0.18e	14.26 ± 0.17	5.76 ± 0.05	3.02± 0.07	0.55 ± 0.01	3.57 ± 0.01	8.93± 0.28
G3. (Standard Treated)	10.24 ± 0.15*	9.82 ± 0.04*	6.54 ± 0.09*	4.55 ± 0.07*	1.28 ± 0.04*	1.32 ± 0.02*	6.68 ± 0.11*
G4. (Curative Treatment)	11.00 ± 0.11*	10.77 ± 0.11*	6.72 ± 0.04*	4.80 ± 0.04*	1.19 ± 0.06*	1.55 ± 0.05*	7.03 ± 0.07*
G5. (Preventive Treatment)	10.54 ± 0.20*	10.26 ± 0.20*	6.66± 0.02*	4.65 ± 0.06*	1.52 ± 0.04*	1.41 ± 0.01*	6.86 ± 0.03*

Each value expressed as the mean ± SD (n=3) one- way ANOVA; -P<0.01 when compared with the normal group; *-p<0.01 hen compared with the lithiatic group.

Table 3: Effect of aqueous extract of *Clitoria ternatea* in serum parameters of lithiatic rats.

	Serum analysis (mg/d)		
	Creatinine	Uric acid	BUN (Blood urea nitrogen)
Group I	0.75 ± 0.01	1.49 ± 0.07	37.61 ± 0.15
Group II	1.73 ± 0.17	4.29 ± 0.14	48.43 ± 0.54
Group III	0.80 ± 0.01*	1.62 ± 0.03*	38.15 ± 1.02*
Group IV	0.88 ± 0.02*	1.92± 0.02*	40.43 ± 0.66*
Group V	0.82 ± 0.02*	1.79 ± 0.03*	39.48 ± 0.49*

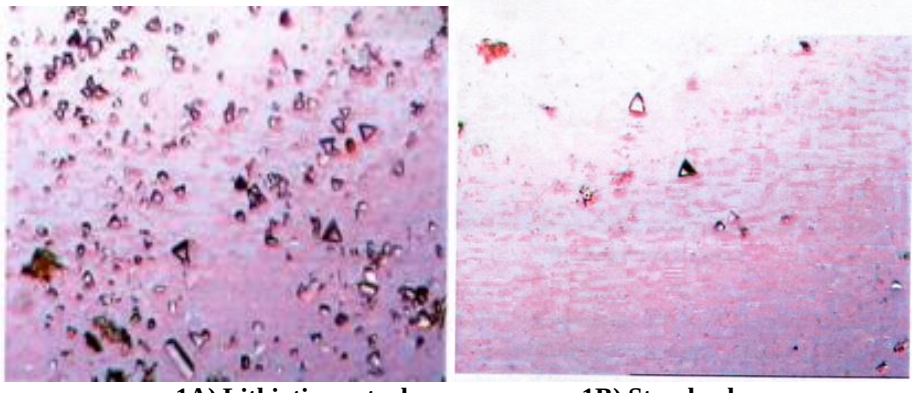
Each value expressed as the mean ± SD (N=6) one – way ANOVA , <0.01 when compared with the normal group, *-p<0.01 when compared with the lithiatic group

Table 4: Diuretic activity of effect of aqueous extract of *Clitoria ternatea.*

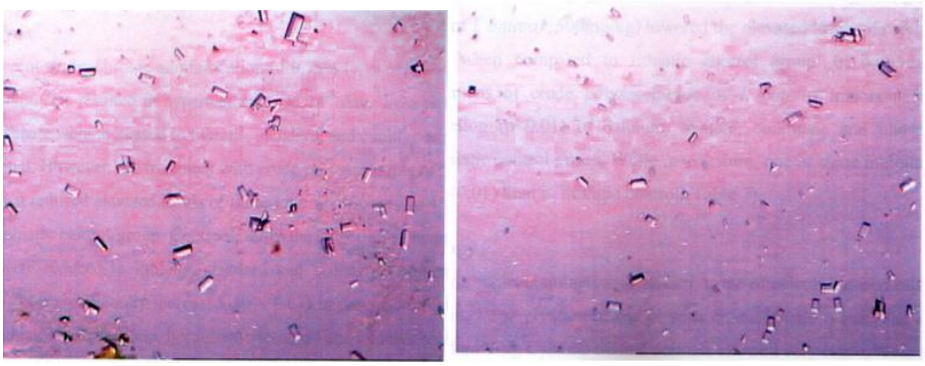
	Water intake (ml/100g//5h)	Urine pH	Urinary Excretion (mg/dl)			
			Na^+	K^+	Cl^-	Diuretic index
Group I	0.51 ± 0.02	6.03 ± 0.06	56.01 ± 1.53	44.15 ± 1.08	38.17 ± 4.8	-
Group II	2.88 ± 0.16	6.71 ± 0.005	111.46 ± 1.20	72.81 ± 0.14	82.20 + 0.44	5.64
Group III	1.27 ± 0.03*	6.67 ± 0.005*	89.09 ± 1.21*	62.95 ± 1.25*	58.87 ± 0.12*	2.47
Group IV	1.74 ± 0.04*	6.69 ± 0.005*	98.25 ± 0.44*	69.50 ± 0.65*	61.16± 0.63*	3.41

Each value expressed as the mean ± SD(n=3)one-way ANOVA, *p<0.01 when compared with the standard group.

Microscopic studies



1A) Lithiatic control **1B) Standard**



1C) Curative regimen **1D) Preventive regimen**

Figure 1: Urine Microscopy (200X) of A) Calculi induced (Untreated) group; b) Cystone treated group; C) Aqueous Extract of *Clitoria ternatea* treated group [CR]; D) Aqueous Extract of *Clitoria ternatea* treated group [PR].

DISCUSSION

Urinary lithiasis is generally the result of an imbalance between inhibitors & promoters in the kidneys. Human kidney stones are usually composed of Calcium oxalate crystals. Rats are the most frequently used animals in Calcium oxalate deposition, as the urinary system of male rats resembles that of humans and earlier studies showed that the amount of stone deposition in female rats was significantly less.[16] The urinary super saturation with stone forming constituents is generally considered to be one of the causative factors in calculogenesis. The aqueous extract of *Clitoria ternatea* contains triterpinoids glycosides etc. it is confirmed by chemical identification test. In the present study, on 14th day the oxalate, phosphate excretion was significantly ($P<0.01$) decreased in the preventive regimen when compared to lithiatic group. The calcium and magnesium excretion in the urine was significantly increased in the preventive group, where as it decreased in the lithiatic group. Urine volume and water intake was significantly increased in lithiatic control group when compared to the normal animals, whereas it was significantly ($p<0.01$) decreased in the preventive group Urinary super saturation with respect to stone forming constituents is generally considered to be one of the causative factors in calculogenesis. Administration of ethylene glycol (0.75%, v/v) to young male albino rats for 14 day period forms renal calculi composed mainly of calcium oxalate. The biochemical mechanisms for this process are related to an increase in the urinary concentration of oxalate. Stone formation in ethylene glycol fed animals is caused by hyperoxaluria, which causes increased renal retention and excretion of oxalate.[17] On 28lh day, the oxalate, phosphate excretion was significantly ($p<0.01$) decreased and the calcium, magnesium excretion levels were significantly ($p<0.01$) increased in both curative and preventive regimens aqueous extract of *Clitoria ternatea* when compared with lithiatic control animals. Following the induction of lithiasis the water intake, urinary volume and composition were found to be altered. In our study also the urinary output and water intake was markedly increased in lithiatic control rats on day 28, however the urinary volumes of AECT and standard treated rats were significantly ($p<0.01$) decreased when compared to that lithiatic group. In urolithiasis, the glomerular filtration rate (GFR) decreases due to the obstruction to the outflow of urine by stones in urinary system. Due to this, the waste products, particularly nitrogenous substances such as urea, creatinine, and uric acid get accumulated in blood.[16] In lithiatic group, the serum levels of creatinine, uric acid, and blood urea nitrogen were elevated, while in curative and preventive groups it was decreased. The analysis of crystalluria after 28 days of treatment with stone inducing agents showed that it is markedly increased in the lithiatic group, while it is decreased in aqueous extract of leaves of *Clitoria ternatea* treatment. In the Diuretic study, the urine volume was elevated in the AECT (250 and 500 mg/kg) treatment when compared to the control animals. The Sodium, potassium, and chloride concentrations were significantly ($P<0.01$) increased in the standard and the AECT treatment. Diuretic index is evaluated to assess the diuretic potential of the plant. The exact mechanism of action of the plants is not known. However it may be due to following actions of the plant *Clitoria ternatea* may be responsible for the antilithiatic activity.

- By increasing using volume pH and anticalcifying activity (diuretic activity) helps in spontaneous passage
- By balancing the inhibition and promotion of crystallization in urine
- Relives the binding mucin of calculi (lithophilic activity)
- By improving renal functions, regulation of oxalate metabolism
- Regulate the crystalloid colloid imbalance and improves the renal function
- Improves renal antioxidant status and cell membrane integrity and prevent recurrence (antioxidant activity)
- Exert significant anti infective action against major curative organisms
- Reveals marked improvement in symptoms of urinary calculi like pain,burning micturation and haematuria (analgesic and antiinflammatory)

However, the treatment of aqueous extract of *Clitoria ternatea* caused diuresis and hastened the process o f dissolving the preformed stones and prevention of new stone formation in urinary system.

All these observations enabled us to confirm the inhibitory and curative potential, of aqueous extract of *Clitoria ternatea* on ethylene glycol induced lithiasis and its diuretic potential.

CONCLUSION

The presented data about various ion concentrations like calcium, oxalate, magnesium, phosphate excretion in urine analysis and creatinine, uric acid, Blood urea nitrogen (BUN) in serum analysis indicates that the administration of aqueous extract of *Clitoria ternatea* leaves to ethylene glycol induced lithiatic rats reduced and prevented the growth of urinary stones, supporting folk information regarding antilithiatic activity of the plant. The mechanism underlying this effect is still unknown, but is apparently related to increased diuresis and lowering of urinary concentrations of stone forming constituents. These finding, thus prompt the necessity for further study to carry out the mechanism of actions related to antilithiatic effect of aqueous extract of *Clitoria ternatea* leaves. By which more effective treatment for lithiasis can be achieved.

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