



Evaluation of antioxidant and diuretic activities of *Clitoria ternatea* leaf extracts in Wistar albino rats

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ABSTRACT

In the present study, the diuretic and antioxidant potential of *Clitoria ternatea* leaf extracts were evaluated. Preliminary phytochemical screening of three solvent extracts [methanol (MCTL), chloroform, and aqueous) of the leaves revealed that the extracts were rich in alkaloids, glycosides, carbohydrates, flavonoids, and saponins. Among the various extracts, optimal antioxidant activity was observed in the MCTL extract. *In vivo* studies, using Wistar albino rats at doses of 150, 300, and 450 mg/kg along with furosemide (20 mg/kg) as standard and Sodium carboxymethyl cellulose (CMC) (2%) as control, evaluated its diuretic activity using parameters like urine volume and urinary electrolytes. Among the three extracts, MCTL extracts at 450 mg/kg concentration possessed reasonable diuretic activity, which was statistically significant ($p < 0.05$) in comparison with furosemide (the standard diuretic drug). Furthermore, antiurolithiatic activity was also shown by the extracts with optimal activity in the MCTL extract showing $73.2\% \pm 2.1\%$ and $48.3\% \pm 3.1\%$ inhibition of nucleation and aggregation, respectively. Based on the findings from this study, it can be suggested that the MCTL extract of the *C. ternatea* leaves show efficacy in conditions such as hypertension and edema, which require flushing out of excess fluids.

INTRODUCTION

The treatment of ailments such as hypertension, nephrotic syndrome, cirrhosis, and heart conditions includes diuretics in order to flush out excessive fluids within the body (Bart *et al.*, 2012). They exert the required effect by regulating the elimination of salts in correspondence with water levels via urine. In hypertensive conditions, for instance, increased salt concentration leads to an elevation of fluid build-up, resulting in increased blood pressure. On the contrary, treatments with diuretic agents reverse this effect by the reduction of fluid build-up in the body, primarily by targeting

the nephrons and regulation of the composition of the filtrate during urine formation (Aliti *et al.*, 2013).

Diuretics commonly administered are thiazide, loop, potassium-sparing osmotic diuretics, and carbonic anhydrase inhibitors. The role of these diuretics is to primarily flush out excess fluids from the body in order to ensure homeostasis. However, considering their chemical origin, they are often associated with certain adverse effects, such as electrolyte imbalance owing to excess urination. Metabolic acidosis, hyperkalemia, hypernatremia, and hypokalemia constitute among a few serious illnesses caused by these drugs. In addition, kidney stones are also a serious issue caused by the administration of potassium-sparing, one of the diuretic agents which causes an elevated calcium excretion in urine. Such drugs are, therefore, administered along with thiazide diuretics in order to lower calcium excretion (Somasekharan *et al.*, 2012). Drugs add up and lead to many other adversities due to their cross reactions. In addition, they pose

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a greater burden to the economy of the country and, therefore, are extremely important to identify a potential diuretic agent with fewer adverse effects. In order to address the side effects associated with synthetic drugs, plant-based medicines are gaining popularity in the recent times. As they are a rich source of phenolic acids, flavonoids, and polyphenols, they have an added advantage of antioxidant properties to fight against the reactive oxygen species (ROS) generated. Natural antioxidants present abundantly in various plant sources inhibit oxidative stress and are capable of protecting against various diseases, including degenerative kidney diseases (Halliwell, 2012).

Clitoria ternatea, also known as butterfly pea or blue pea, is a well-known herb used in traditional medicine from the family Fabaceae. Various parts such as the shoots, roots, leaves, and seeds are known for various traditional uses, such as an anxiolytic, memory enhancer, anticonvulsant, anti-stressor, nootropic, antidepressant, and tranquilizing and sedative agent (Gupta *et al.*, 2010). *Clitoria ternatea* possesses potential health beneficiary properties, such as antioxidant, anticancer, antipyretic, anti-inflammatory, hypolipidemic, respiratory, cardiovascular, immunological, analgesic, insecticidal, and others (Mukherjee *et al.*, 2008). This literature review showed that the alcoholic extracts of the roots of *C. ternatea* revealed diuretic activity in dogs (Piala *et al.*, 1962). No study has been carried out using the leaf extract of the plant; therefore, the present study focused to evaluate the diuretic activity of the leaf extract from *C. ternatea*.

MATERIALS AND METHODS

Plant material

Leaves of *C. ternatea* (CT) were collected from Mysore, Karnataka, India, in Feb 2018 and were authenticated at the Pharmacognosy Department, JSS College of Pharmacy, Mysore. A voucher specimen has been deposited and preserved in the herbarium of JSS College of Pharmacy, Mysore.

Preparation of the plant extract

Shade-dried leaves of *C. ternatea* were subjected to successive extraction using various solvents [chloroform (CCTL), methanol (MCTL), and water] in increasing order of polarity. MCTL (100%) and CCTL (100%) extracts were extracted with the Soxhlet apparatus (De Castro *et al.*, 1998), whereas the aqueous (ACTL) extract (1:10 w/v) was prepared using the maceration method (by immersing the dried powder of the leaf in water inside an air-tight container) for 7 days at room temperature (Zimman *et al.*, 2002). The extracts were concentrated using the rotary evaporator and dried under vacuum. The percentage yield of MCTL, CCTL, and ACTL extracts was 11.69%, 9.48%, and 10.32%, respectively.

The experiments were in accordance with the guidance from the Institutional Animal Ethics Committee of JSS College of Pharmacy, JSS AHER, Mysuru (JSSMC/Pharma/P5-260/2017). Male Wistar rats (8–10 weeks old) weighing 200–250 g were considered for the study and handled as per the guidelines issued by the Institutional Animal Ethics Committee, JSS College of Pharmacy, Mysuru, Karnataka. After procuring them from a local breeder, the animals were acclimatized to the experimental conditions and maintained at 23°C ± 3°C temperature and 50% ± 5% humidity inside polypropylene cages with paddy husk as

bedding material. Each cage contained six animals and all were provided with standard food pellets and water *ad libitum*.

Chemicals and reagents used

Folin–Ciocalteu (FC) reagent, sodium acetate trihydrate buffer, 2,4,6-tripyridyl-S-triazine, hydrochloric acid (HCl), 20 mM ferric chloride solution, 2,2-diphenylpicrylhydrazyl (DPPH), Normal saline, sodium CMC 2%, and furosemide 20 mg were used.

Phytochemical analysis

Phytochemical analysis was carried out using standard tests (Harborne, 1973). The MCTL, CCTL, and ACTL extracts of the leaf extract of *C. ternatea* were subjected to preliminary phytochemical analyses.

Estimation of total phenolic content (TPC) by FC reagent (FC method)

The total phenol content was estimated using the FC method, which is estimated by a blue-colored phosphotungstic-phosphomolybdenum complex. This complex was measured at an absorbance of 765 nm (Shan *et al.*, 2005).

Measurement of the antioxidant activity using the ferric-reducing antioxidant power (FRAP) method

The antioxidant potential of the extracts was evaluated using the FRAP assay as described by Benzie and Strain (1999). The FRAP assay is primarily carried out to assess the reduction of ferric 2, 4, 6-tri-pyridyl-s-triazine complex to its ferrous form under conditions, such as low pH. This reduction is measured as a function of change in absorbance at 593 nm.

Antioxidant capacity using the DPPH scavenging assay

The antioxidant potential of the extracts was evaluated using the DPPH radical scavenging capacity as described previously with slight modifications (Patel and Patel, 2011). In brief, five different concentrations of the extract (0.5 ml) taken along with 3.5 ml DPPH solution was incubated for a period of 30 minutes in dark at room temperature (RT). The absorbance of the sample, 95% ethanol or water, was recorded at 536 nm. The activity was calculated using the following formula:

$$\% \text{ scavenging activity} = (A_0 - A_1) / A_0 \times 100$$

where A_0 is the absorbance of the blank and A_1 is the absorbance of the standard or test.

Diuretic activity

The diuretic activity of the extracts was evaluated using the Lipschitz model (Lipschitz *et al.*, 1943). The experiment was carried out in five groups with six animals in each group. All the rats were fasted and deprived of water for 18 hours before the commencement of the experiment. Group 1 (control) was treated with vehicle (sodium CMC 2% in normal saline), whereas group 2 was treated with a standard dose of furosemide 20 mg/kg (in normal saline). Group 3 a, b, c; Group 4 a, b, c; and Group 5 a, b, c were orally treated with three different extracts of MCTL, ACTL, and CCTL with dosage of 150 mg, 300 mg, and 450 mg for each extract, respectively. The experimental animals were then housed in diuretic cages (designed to separate urine and feces), with two

rats in each cage, containing a netted floor and maintained at room temperature ($25^{\circ}\text{C} \pm 0.5^{\circ}\text{C}$). However, the administration of water and food was restricted during this period. Urine was collected in a conical flask placed below the polythene funnel of the diuretic cage. Extreme care was taken to avoid contamination of urine with fecal matter. 5 hours post-dosing, urine was collected from the cages and assessed for its volume, ion concentrations of Na^+ , K^+ (cations) using flame photometer, and Chloride anion (Cl^-) (anions) using Argentometric titration. The determined volume of urine was in terms of ml/5 hours, whereas the concentration of electrolytes was expressed as mmol/l. The diuretic index and diuretic activity (Vogel *et al.*, 2008) were calculated as follows: Diuretic index = Urinary excretion of treated group / Urinary excretion of control group

Diuretic activity = Diuretic action of test drug / Diuretic action of standard

Nucleation assay

Spectrophotometric assay was carried out in order to evaluate the nucleation inhibition of CaOx crystals by the extracts and standard cystone. The addition of CaCl_2 and $\text{Na}_2\text{C}_2\text{O}_4$ to synthetic urine led to crystallization. For this, solutions of 4 mmol/l CaCl_2 and 50 mmol/l $\text{Na}_2\text{C}_2\text{O}_4$ were prepared using 0.05 mol/l Tris buffer in 0.15 mol/l NaCl under physiological temperature and pH. Different concentrations of the extracts (400, 600, 800, and 1,000 $\mu\text{g/ml}$) were taken and 1 ml each was mixed with 3 ml CaCl_2 solution, followed by the addition of 3 ml $\text{Na}_2\text{C}_2\text{O}_4$ solution. Final mixtures were incubated for 1, 2, and 3 hours at 37°C . The absorbance of the mixtures was then measured at 620 nm wavelength for 3 different hours. Percent inhibition of nucleation by each fraction was calculated by using the following formula:

$$\% \text{ Inhibition} = [1 - (\text{OD test} / \text{OD control})] \times 100$$

Aggregation assay

The effect of various solvent fractions on CaOx crystal aggregation was determined by means of an aggregation assay. CaCl_2 and $\text{Na}_2\text{C}_2\text{O}_4$ solutions (50 mmol/l each) were mixed together, heated to 60°C in a water bath for 1 hour, and then incubated overnight at 37°C to prepare seed CaOx crystals. After drying, CaOx crystal solution (0.8 mg/ml) was prepared in a 0.05 mol/l Tris-HCl and 0.15 mol/l NaCl buffer (pH 6.5). Different concentrations of the extracts (400, 600, 800, and 1,000 $\mu\text{g/ml}$) from each solvent fraction were added to 3 ml CaOx solution, vortexed and then incubated at 37°C for 3 different hours (1, 2, and 3 h). OD of the final mixtures was then read at 620 nm wavelength and percent inhibition of aggregation was then calculated as described for nucleation assay as follows: % Inhibition = $[1 - (\text{OD test} / \text{OD control})] \times 100$

Statistical analysis

The experiments were carried out in triplicates and the results were expressed as mean \pm SD. Statistical comparisons between the control and treatment groups were conducted by one-way analysis of variance (ANOVA), followed by Tukey's *post-hoc* and Dunnett's tests by employing Statistical Package for the Social Sciences software (version 21.0, Chicago, IL). Graph Pad PRISM software (version 4.03) was used for calculating Half-maximal inhibitory concentration (IC_{50}) values.

RESULTS AND DISCUSSION

Preliminary phytochemical screening using various extracts from *C. ternatea* leaves showed the presence of flavonoids, alkaloids, saponins, glycosides, and carbohydrates. Several studies have reported optimum extraction of the primary phytochemicals in the MCTL extract, which is in agreement with our study (Shan *et al.*, 2005; Patel and Patel, 2011).

Phenolic content represented in terms of gallic acid equivalents demonstrated that among the three extracts, the highest concentration was seen in the MCTL extract of *C. ternatea* (MCTL = 22.63%), followed by the ACTL extract (ACTL = 20.92%) and CCTL extract (CCTL = 16.83%). The TPC of different extracts is summarized in Table 1. The antioxidant potential of the extracts was assessed by the FRAP and DPPH assays. In the present study, with respect to the FRAP assay, optimal reduction of ferric ion was observed in MCTL ($R^2 = 0.9838$), that was followed by CCTL ($R^2 = 0.9764$) and ACTL ($R^2 = 0.9789$). The antioxidant activity of these extracts is on par with that of the standard drug (ascorbic acid) in a dose-dependent manner. The antioxidant potential of most plant extracts is not a new concept. However, any plant extract with antioxidant property proves beneficiary because it not only possesses the actual disease-preventing activity but also protects from other secondary damages caused by the condition due to the generation of ROS (Ramu *et al.*, 2014). Therefore, in agreement with previous studies, *C. ternatea* leaf extracts showed good antioxidant activity, which can be proven to be an adjunct to the able diuretic property of the extract (Benzie and Strain, 1999).

The DPPH-reducing ability was represented as IC_{50} , which was determined by evaluating the substrate concentration required for 50% loss of the DPPH activity. Diverse concentrations of extracts (MCTL, CCTL, and ACTL) demonstrated a dose-dependent antioxidant activity, with the MCTL showing the highest (IC_{50} : 35.5 $\mu\text{g/ml}$), when compared to that of CCTL (IC_{50} : 45.3 $\mu\text{g/ml}$) and ACTL (IC_{50} : 41.1 $\mu\text{g/ml}$). The antioxidant activity of the standard in this study (IC_{50} : 21.01 $\mu\text{g/ml}$) was compared to that of the extracts, with MCTL showing optimum among the three extracts. The antioxidant activity with both assays (FRAP and DPPH) could be summarized as vitamin C > MCTL > ACTL > CCTL. The free radical scavenging capacity and antioxidant activities are summarized in Table 2.

Lipschitz's method was used to carry out the diuretic activity. Based on the results in Patel and Patel's (2011) study, it was suggested that values ranging >1.50 showed significant diuretic activity, whereas 1.00–1.50 were moderate, 0.72–0.99 showed mild, and <0.72 demonstrated no diuretic activity. The results from our study showed that all the extracts revealed diuretic properties in a dose-dependent manner and were comparable with that of the standard drug (furosemide). Optimum diuretic activity was observed in MCTL at a maximum dose of 450 mg/

Table 1. TPC of different extracts.

Extracts	TPC
MCTL	22.63 \pm 0.19
CCTL	20.92 \pm 0.14
ACTL	16.83 \pm 0.06

Data are expressed in mean values of $n = 3 \pm \text{SEM}$. All data are analyzed using Students t-test and compared with other groups ($*p < 0.05$).

Table 2. Free radical scavenging and antioxidant activity by FRAP and DPPH assays.

Extracts	Conc. ($\mu\text{g/ml}$)	FRAP units	DPPH % Inhibition	DPPH IC_{50} ($\mu\text{g/ml}$)
MCTL	5	95.2 \pm 0.15	15.9 \pm 0.4	35.5 $\mu\text{g/ml}$
	25	183.1 \pm 0.3	34.2 \pm 0.28	
	50	865.69 \pm 2.5	62.1 \pm 1.57	
	100	1,559.4 \pm 0.9	78.5 \pm 0.4	
CCTL	5	88.1 \pm 0.23	13.3 \pm 0.2	=43.3 $\mu\text{g/ml}$
	25	171.1 \pm 0.64	22.2 \pm 0.14	
	50	789.87 \pm 1.9	48.3 \pm 0.29	
	100	1,428.9 \pm 0.7	66.5 \pm 0.5	
ACTL	5	98.2 \pm 1.5	15.2 \pm 0.23	=41.1 $\mu\text{g/ml}$
	25	182.1 \pm 0.3	32.3 \pm 0.68	
	50	864.5 \pm 2.5	59.8 \pm 1.57	
	100	1,552.7 \pm 1.2	70.6 \pm 1.02	

All data were analyzed by one-way ANOVA (followed by post-hoc Turkey's multiple comparison tests). All the values are mean \pm SEM, where $p < 0.05$ is compared to the standard.

Table 3. Diuretic activity of MCTL, CCTL, and ACTL extracts of *Clitoria ternatea* leaves.

Groups	Mean urine volume (ml)	Urinary electrolyte excretion			Diuretic index	Diuretic activity
		Na^+ (meq/l)	K^+ (meq/l)	Cl^- (meq/l)		
Control	12.18 \pm 1.02	112.80 \pm 0.21	51.15 \pm 0.43	62.07 \pm 0.17	1.00	0.50
Standard	23.34 \pm 0.59**	191.32 \pm 0.40**	93.10 \pm 0.29**	128.14 \pm 0.17**	1.99**	1.00**
MCTL 150 mg	15.58 \pm 0.09*	121.48 \pm 0.04	62.32 \pm 0.45	82.82 \pm 0.31*	1.27*	0.63*
MCTL 300 mg	17.51 \pm 2.08*	128.71 \pm 0.03*	70.07 \pm 1.29*	98.24 \pm 0.33*	1.43*	0.71*
MCTL 450 mg	20.73 \pm 0.05*	142.03 \pm 0.21*	82.92 \pm 0.29**	121.71 \pm 0.50**	1.70**	0.85**
CCTL 150 mg	12.30 \pm 0.01	114.15 \pm 0.02	53.98 \pm 0.34	63.20 \pm 0.27	1.00	0.50
CCTL 300 mg	13.49 \pm 0.07	118.63 \pm 0.05	49.23 \pm 0.21	74.78 \pm 0.46	1.09	0.54
CCTL 450 mg	18.23 \pm 0.05*	123.38 \pm 0.08	72.92 \pm 0.22*	108.22 \pm 0.08**	1.49*	0.74*
ACTL 150 mg	15.21 \pm 0.02*	119.23 \pm 0.02	60.86 \pm 0.45	70.49 \pm 0.54	1.24*	0.62*
ACTL 300 mg	16.78 \pm 0.06*	126.88 \pm 1.04*	67.15 \pm 0.21*	79.62 \pm 0.49*	1.37*	0.68*
ACTL 450 mg	20.47 \pm 0.02*	139.95 \pm 0.01*	86.88 \pm 0.27**	118.31 \pm 0.15**	1.68**	0.84**

Values are expressed as mean \pm SEM, $n = 6$. In comparison to the control group = one-way ANOVA followed by Dunnett's post-hoc test; * $p < 0.05$ compared to control group = one-way ANOVA followed by Dunnett's post-hoc test; and ** $p < 0.01$ compared to control group = one-way ANOVA followed by Dunnett's post-hoc test.

kg (1.70), followed by ACTL (1.68) and CCTL (1.49) in a dose-dependent manner, as against the standard drug (1.99). The mean urine volume and electrolyte concentrations (Na^+ and K^+) were elevated in a dose-dependent manner in all the extracts tested, but only moderate excretion of Cl^- ions was seen. The diuretic activity of all the three extracts is summarized in Table 3. The diuretic activity was maximum in the MCTL extract over that of either ACTL and CCTL extracts, as well as the standard drug (MCTL > ACTL > CCTL).

The urolithiatic abilities of the polyherbal combination, cystone, led to its use as a standard drug in our study. The extracts obtained from various solvents, ranging from polar to non-polar, ensured the extraction of compounds with diversified solubility. In the present study, the three extracts showed significant inhibition at $>800 \mu\text{g/ml}$. At $1,000 \mu\text{g/ml}$, the three extracts showed an appreciable percent inhibition. CaOx crystal nucleation studies revealed that the percent inhibition exerted by MCTL (73.2 ± 2.1), CCTL (63.5 ± 1.1), and ACTL (48.2 ± 3.4) was optimal at the longest duration (3 h) and was on par with the standard drug cystone (8.352 ± 2.2). Inhibition using CCTL did not vary greatly with respect to time, but ACTL was optimal at 1 hour. Also, MCTL, which was on par with the standard drug cystone, was optimal at 3 hours (Table 4).

The effect of different solvent extracts on calcium oxalate crystal aggregation was also evaluated. The optimal activity was observed with the MCTL treatment for 3 hours but was relatively lower than the standard drug cystone. Nevertheless, the inhibition exerted by the MCTL was 48.3 ± 3.1 , which was remarkable compared to all the solvent extracts (Table 4).

Furthermore, the CaOx crystals were analyzed using microphotography studies, which revealed that the MCTL extract resulted in the formation of more calcium oxalate dehydrate crystals than COM crystals. Such a formation is beneficial because calcium oxalate monohydrate crystals render greater damage to the epithelial cells of the renal system. The inhibition of nucleation and aggregation shown by the MCTL treatment, therefore, proves beneficiary as possessing antiurolithiatic activity. Diuretic activity is primarily important in conditions such as hypertension, liver cirrhosis, and certain types of edema; therefore, it is advantageous as a pre-treatment before testing with various fluids as screening agents. In our study, the diuretic activity of the extracts was on par with the standard drug furosemide at the highest concentration of the MCTL. This was accompanied by the urinary excretion of electrolytes. Previous studies have reported that greater excretion of sodium over that of potassium is an indicator of safety profile of diuretic agents (Maghrani *et al.*,

Table 4. Effect of MCTL, CCTL, and ACTL extracts of *Clitoria ternatea* on nucleation and aggregation of calcium oxalate crystals at 1,000 µg/ml.

Solvent	Nucleation assay (%inhibition)			Aggregation assay (%inhibition)		
	1 hour	2 hours	3 hours	1 hours	2 hours	3 hours
Cystone	58.50 ± 3.41d	70.50 ± 30.16c	83.50 ± 2.25a	48.45 ± 3.65 d	58.25 ± 3.40 c	78.35 ± 2.52a
MCTL	68.15 ± 3.63c	70.60 ± 1.10c	73.25 ± 2.10b	28.50 ± 3.91	37.60 ± 4.95e	48.34 ± 3.15d
CCTL	58.30 ± 3.42d	60.50 ± 1.15d	63.18 ± 1.15d	13.30 ± 2.05	24.10 ± 4.32	29.25 ± 4.25
ACTL	63.10 ± 2.10d	64.60 ± 1.72d	48.25 ± 3.40c	20.57 ± 4.48	31.01 ± 4.15	43.60 ± 3.75

All values are expressed in mean ± standard error of the mean (SEM).

* $p < 0.05$ is considered as statistically significant where $a > b > c > d$.

2005; Sripanidkulchai *et al.*, 2001). In our study, the excretion of sodium was more than that of the other two electrolytes (potassium and chlorine), thereby agreeing with the previous findings. Increased excretion of these electrolytes is mainly due to the inhibitory role of the extract on carbonic anhydrase. Therefore, our study suggests that one of the possible diuretic potential operates by inhibition of carbonic anhydrase, which is in agreement with the report of Vogel (2007).

CONCLUSION

The extracts possessed remarkable diuretic properties with the MCTL extract showing optimal activity among the three solvent extracts. This may be due to the greater ability of the extraction of saponins, flavonoids, and phenolic acids by MCTL when compared to other solvents. The extract also showed potential antiurolithiatic activity observed by the inhibition of nucleation and aggregation of CaOx crystals. In addition, potential antioxidant activity exerted by the extract on the FRAP and DPPH upholds the beneficiary potential of the *C. ternatea* leaf extract in several diseases, including hypertension and edema.

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AUTHOR CONTRIBUTIONS

All authors made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; took part in drafting the article or revising it critically for important intellectual content; agreed to submit to the current journal; gave final approval of the version to be published; and agree to be accountable for all aspects of the work.

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CONFLICTS OF INTEREST

The authors report no conflicts of interest in this work.

ETHICAL APPROVAL

This study was approved by the Institutional Animal Ethics Committee of JSS College of Pharmacy, JSS AHER, Mysuru, India. (JSSMC/Pharma/P5-260/2017).

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