

Research Article

Anti Urolithiatic and anti hyperlipidemic activity of *Coleus aromaticus* An explanation of the underlying mechanisms

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Abstract

Leaves paste of *Coleus aromaticus* is used as a traditional remedy for urolithiasis in India. In the present study, the anti urolithiatic activity of *Coleus aromaticus* was investigated in ethylene glycol induced urolithiatic rats. There was a significant increase in the levels of calcium oxalate crystals in the kidneys as well as lipid levels in the blood serum. Treatment with hydro alcoholic extract of *C. aromaticus* leaves (CALHAE) significantly reduced cholesterol levels at 300 and 600 mg/kg, and triglyceride levels at 600 mg/kg in urolithiatic rats. Histopathological reports confirmed that chronic administration of CALHAE (300 and 600 mg/kg) diminished number of calcium oxalate crystals in kidneys. CALHAE has shown reduction in thiobarbituric acid reactive substances (TBARS) in urolithiatic rats. Moreover, CALHAE showed potent in vitro antioxidant activity in DMPD, ABTS radicals (MnO₂ method). Results from these studies support the safe and effective use of *C. aromaticus* leaves for urolithiasis treatment.

Keywords: *Coleus aromaticus*, Calcium oxalate crystals, Hypolipidemic activity, Antioxidant activity

Introduction

Coleus aromaticus is commonly known as country borage. It is a large succulent aromatic perennial herb with hispidly villous or tomentose fleshy stem. Leaves are simple opposite, broadly ovate, crenate, fleshy and very aromatic. The leaves of *C. aromaticus* are often eaten raw with bread and butter and it is used for seasoning meat dishes and in food products. Decoction of its leaves is used in treating chronic cough and asthma. Experimentally, leaf oil was exhibited activity against microorganisms like fungi and bacteria [1]. Both the aqueous and hydro

alcoholic extracts of *C. aromaticus* stabilizes mast cells in the rat mesenteric tissue [2].

Urolithiasis is a common painful condition of multifactorial ethiopathogenesis, and calcium oxalate (CaOX) is the commonest component of upper urinary tract calculi in economically developed countries, including North America, Europe, Australia, and Japan. Many epidemiological factors, including age, sex, heredity, occupation, body size, social class, affluence, geographic location, climate, diet and fluid intake, have been identified as playing roles in kidney stone disease [3]. Oxalate is found in many dietary sources, including spinach, rhubarb, straw berries, and tea, it is the major kidney stone

forming constituent and induces lipid peroxidation and alters the lipid profiles, so for stone formation, one of the mechanisms was a systemic abnormality in lipid metabolism. Stone formers are significantly associated with several CHD risk factors [4]. Hyperlipidemia associated with the nephritic syndrome is a complex disorder involving abnormalities most likely induced by the glomerular barrier defect [5] Furthermore, oxalate increases the production of free radicals, which can induce a cell death process, crystal deposition in the renal tubules, which leads to growth of calcium oxalate stones. Human, animal, explants and in vitro studies indicate that raised oxidative stress is often present in urolithiasis and oxalate per se promotes oxidative stress, which is substantially retarded by antioxidants [6].

Urolithiasis is largely a recurrent disease with a relapse rate of 50% in 5–10 years, thereby exists with substantial economic consequences and a great public health importance [7]. Endoscopic stone removal and extracorporeal shock wave lithotripsy have revolutionized the treatment of urolithiasis but do not prevent the likelihood of new stone formation [8]. Various therapies including thiazide diuretics and alkali-citrate are being used in attempt to prevent recurrence of hypercalciuria and hyperoxaluria induced calculi but scientific evidence for their efficacy is less convincing. The current study elucidates the anti urolithiatic and possible hypolipidemic activity of *C.aromaticus* on ethylene glycol induced urolithiasis in rats.

Materials and Methods

Plant collection and extraction

Fresh leaves of *C.aromaticus* were collected from Herbal Garden and Research center of Acharya N.G. Ranga Agricultural University, Rajendra nagar, Hyderabad, India. It was identified and authenticated by Dr. R. Mohan Rao, Senior Scientist, Office incharge of Herbal garden and Research center. The fresh leaves were dried under shade and then powdered with mechanical grinder. The powder was packed in soxhlet

column and extracted with methanol and distilled water in the ratio of 1:1 for 16 h at 40 °C. The extract was concentrated using rotary flash evaporator under reduced pressure and the yield was 24% w/w. The dried extract was stored in airtight container in refrigerator below 8 °C.

Experimental animals

Male wistar rats, weighing between 150-200 g were used. These animals were obtained from animal house facility of Shadan institute of medical sciences and Research Institute. They were kept in polypropylene cages with not more than five animals per cage and maintained under standard laboratory conditions (temperature 25 ± 2 °C) in a well-ventilated room under hygienic condition, with a dark and light cycle of (12 h each). The animals were fed with extruded pelleted rat/mouse feed manufactured by Rayans Hyderabad Pvt. Ltd., were provided *ad libitum*. The study was approved by Shadan College of Medical Sciences and Research Institute, Shadan College of Pharmacy, Institutional Animal Ethical Committee.

Chemicals and Reagents

Ethylene glycol, ammonium chloride, trichloroacetic acid, thiobarbituric acid, linoleic acid, ammonium thiocyanate, ferrous chloride, butylated hydroxy toluene, α -tocopherol, ammonium molybdate, ascorbic acid, ABTS ([2,2-azinobis (3-ethyl benzothiazoline-6-sulfonic acid) diammonium salt]) and DMPD (N,N-dimethyl-p-phenylene diamine) were obtained from Sigma Chemical Company, St. Louis, MO, and USA. Hydrochloric acid, potassium chloride, manganese dioxide, ferric chloride, sulphuric acid and sodium phosphate were purchased from NICE Chemicals Pvt. Ltd., Cochin. Disodium hydrogen phosphate, potassium dihydrogen phosphate and sodium hydroxide were purchased from Merck (India) Ltd., Mumbai.

In vitro total antioxidant capacity

Total antioxidant capacity was determined by following a procedure similar to that of [9]. Different concentrations of CALHAE and

ascorbic acid (ASC) are mixed separately with 3 ml of reagent solution (0.6 M sulphuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate), incubated at 95 °C for 90 min, cooled to room temperature and absorbance was measured at 695 nm. Plotting the absorbance against ASC concentration gave the linear curve with a correlation coefficient ($r^2 = 0.996$). Total antioxidant capacity was expressed as the number of equivalents of ascorbic acid (ASC) using standard plot.

***In vitro* ABTS⁺ radical inhibition (MnO₂ method)**

Antioxidant activity was determined following a procedure similar to that of [10]. The ABTS⁺ radical cation was generated by filtering a solution of ABTS in PBS through manganese dioxide powder. Excess manganese dioxide was removed from the filtrate by passing it through a 0.2 µ syringe filter, this solution was diluted in 5 mM PBS pH 7.4, adjusted to an absorbance of 0.700 at 734 nm and pre-incubated at room temperature prior to use for 2 h. 1 ml of the ABTS⁺ solution & 200 µL solution of antioxidants were vortexed for 30 s, centrifuged for 60 s at 10,000 rpm. Absorbance was recorded at 734 nm. Solvent blanks were run in each assay. The antioxidant activity of the substances was calculated by determining the decrease in absorbance at different concentrations by using the following equation.

% ABTS⁺ scavenging activity = [(Control absorbance - Extract absorbance) / (Control absorbance)] * 100

***In vitro* linoleic acid peroxidation inhibition, Ferric thiocyanate (FTC) method**

The standard method as described by Kikuzaki and Nakatani [11] was used. A mixture of 4 mg of plant extract in 4 ml of absolute ethanol, 4.1 ml of 2.52% linoleic acid in absolute ethanol, 8.0 ml of 0.05 M phosphate buffer (pH 7.0), and 3.9 ml of water was placed in a vial with a screw cap and then placed in a dark oven at 40 °C. To 0.1 ml of this solution 9.7 ml of 75% ethanol and 0.1 ml of 30% ammonium thiocyanate were added. Precisely 3 min after the addition of 0.1 ml of 0.02 M ferrous chloride in 3.5% HCl to the reaction mixture, the absorbance of red color was

measured at 500 nm every 24 h until one day after the absorbance of control reached its maximum. Butylated hydroxy toluene (BHT) and α-tocopherol were used as positive controls, while a mixture without a plant sample was used as the negative control.

***In vitro* DMPD radical inhibition**

DMPD, 100 mM was prepared by dissolving 209 mg of DMPD in 10 ml of deionized water, 1 ml of this solution was added to 100 ml of 0.1 M acetate buffer, pH 5.25, and the colored radical cation (DMPD⁺) was obtained by adding 0.2 ml of a solution of 0.05 M ferric chloride (final concentration 0.1 mM). 1 ml of this solution was directly placed in a 1 ml plastic cuvette and its absorbance at 505 nm was measured. An optical density of 0.900 absorbance was obtained and it represents the uninhibited signal. The optical density of this solution, which is freshly prepared daily, is constant up to 12 h at room temperature. Standard solutions of the different antioxidant compounds were prepared as follows: 1 mg/ml of ascorbic acid was prepared by dissolving 0.1 g of ascorbic acid in 100 ml of deionized water. Extract solutions concentration was 6 mg/ml. 50 µl of extract solution was added to 1 ml of DMPD solution, after that mixed properly for 10 min and recorded absorbance at 505 nm [12].

% DMPD scavenging activity = [(Control absorbance - Extract absorbance) / (Control absorbance)] * 100

***In vivo* experimental design**

Antiuro lithiatic activity of CALHAE was determined by using ethylene glycol induced hyperoxaluria model as described with some modifications was used to assess the antiuro lithiatic activity in albino rats. Animals were divided into five groups containing six animals in each. Group I served as control and received free access regular rat food and drinking water. Ethylene glycol (0.75%) in drinking water was provided to Groups II–V for induction of renal calculi for 28 days. Group III received standard antiuro lithiatic drug cystone (500 mg/kg) from day 15 to 28 [13]. Groups IV and V served as curative regimen (CR). Group IV received CALHAE (300 mg/kg) and Group V

received CALHAE (600 mg/kg) at dose volume of 2 ml/kg through oral route of administration from day 15 to 28. On day 0, 7 and 15 using metabolic cages 24 h urine of ethylene glycol treated rats was collected, centrifuged at (2,500 rpm for 10 min) and subjected to microscopic analysis for the conformation of the stone formation [14].

Blood samples were collected on day 28 through retro orbital plexus of rats under ether anesthesia using a glass capillary tube after 12 h fasting and the blood was centrifuged (4,000 rpm for 10 min) to get serum. Biochemical estimation of total cholesterol (TC), HDL, LDL and triglyceride (TG) was done with the help of kit-based methods by using semi autoanalyser (Star plus).

The abdomen was cut opened to remove both kidneys from each animal. Isolated kidneys were cleaned off from extraneous tissue and rinsed in ice-cold physiological saline. The right kidney was fixed in 10% neutral buffered formalin, processed in a series of graded alcohol and xylene, embedded in paraffin wax, sectioned at 5 μ m and stained with H and E (Haematoxylin and Eosin) for histopathological examination. The slides were examined under light microscope to study light microscopic architecture of the kidney and calcium oxalate deposits. The left kidney was finely minced and 10% homogenate was prepared in Tris-HCl buffer (0.02 mol/l, pH 7.4) and was

The results of biochemical estimation were reported as mean \pm standard error of mean (S.E.M). The data was analysed by (Graph Pad Prism, 4 Version) One-way analysis of variance (ANOVA). Differences among the means were analysed by Dunnett's posthoc test and $p < 0.05$ was considered significant.

Results

In vitro linoleic acid peroxidation inhibitory activity

The antioxidant activities of CALHAE in preventing the peroxidation of linoleic acid, as measured by thiocyanate method, are shown in **Table 1**. Absorbance of control increased up to 1.845 at 168 h. This is due to oxidation of linoleic acid, generating linoleic acid hydroperoxides, which leads to many secondary oxidation products [16]. The oxidized products (namely, linoleic acid hydroperoxides) react with ferrous chloride to form ferric chloride, then to ferric thiocyanate (blood red color). In the presence of CALHAE and BHT, oxidation of linoleic acid was very slow. Hence, the color development is slow.

In vitro total antioxidant capacity (TAC)

The assay is based on the reduction of molybdate-VI (MoVI) to molybdate-V (MoV) by the extract and subsequent formation of a green phosphate/MoV complex in acidic pH. Activity was expressed as the number of equivalents of

Table 1:

CALHAE conc. (mg/mL)	Absorbance of the reaction mixture at 500 nm in different days						
	Day1	Day2	Day3	Day4	Day5	Day6	Day7
1 mg	0.212	0.235	0.243	0.244	0.248	0.251	0.26
2 mg	0.178	0.198	0.205	0.21	0.219	0.221	0.228
3 mg	0.153	0.18	0.184	0.186	0.195	0.201	0.206
BHT-1 mg	0.122	0.124	0.127	0.129	0.131	0.135	0.138
Control	0.388	0.669	0.807	0.985	1.151	1.412	1.845

used to estimate thiobarbituric acid reactive substances (TBARS) [15].

Statistical analysis

ascorbic acid (ASC) using standard plot. Here CALHAE was found to possess potent antioxidant capacity (reduction of MoVI to MoV) of 8 μ g of ASC equivalents per 175 μ g of the CALHAE (**Table 2**).

Table 2:

Method	CALHAE (IC ₅₀ in µg)	Ascorbic acid (IC ₅₀ in µg)
Total antioxidant assay - number of equivalents of ascorbic acid	175	8
ABTS radical inhibition	9.1	3.7
DMPD radical inhibition	148	33

***In vitro* ABTS⁺ radical inhibitory activity**

ABTS (2, 2'-azinobis-3-ethylbenzothiozoline-6-sulphonic acid) assay is based on the scavenging of light by ABTS radicals. An antioxidant with an ability to donate a hydrogen atom will quench the stable free radical, a process that is associated with a change in absorption that can be followed spectrophotometrically. The relatively stable ABTS radical has a green color and was quantified spectrophotometrically at 734 nm. Here CALHAE showed an appreciable antioxidant activity of 50 % at 9.1 µg/ml, while ascorbic acid shown 50% antioxidant activity at 3.7 µg/ml (Table 2).

***In vitro* DMPD radical inhibitory activity**

Mainly DMPD^{•+} assay was based on generation of a violet colored radical cation resulting from the reaction of DMPD with FeCl₃ at an acidic pH. The extent of decolorization on addition of standard/sample solutions to DMPD^{•+} was determined in terms of percentage inhibition. The method is applicable to the study of both water-soluble and lipid-soluble antioxidants, pure compounds, and food extracts. The CALHAE showed a concentration-dependent antiradical activity by inhibiting DMPD radical with an IC₅₀ value of 148 µg/ml and ascorbic acid IC₅₀ was 33 µg/ml (Table 2).

Anti urolithiatic effect of CALHAE in ethylene glycol induced urolithiatic rats

Administration of ethylene glycol induced urolithiasis and resulted in elevation of lipid

parameters like serum cholesterol, triglyceride, HDL and LDL (p<0.01). CALHAE significantly reduced the cholesterol at 300 mg/kg (p<0.05) and 600 mg/kg (p<0.01) dose levels. It also reduced triglyceride levels in urolithiatic rats, but statistical significance was reached at 600mg/kg dose (p<0.05) (Table 3). Both cystone and CALHAE had no effect on HDL and LDL levels (Table 4). In rats, ethylene glycol induced urolithiasis and related oxidative changes. Both CALHAE and cystone have reduced the thiobarbituric acid reactive substances significantly (p<0.01) in the kidney (Table 4). Vehicle treated rat kidney tissue was devoid of calcium oxalate crystals. In contrast Ethylene glycol induced urolithiasis rat kidney which was evident through shown the deposition of calcium oxalate crystals (Figs. 1a, 1b) in medulla region and in renal tubules, which are large polyclonal crystals. The urolithiatic rats treated with CALHAE (300 and 600 mg/kg) have shown reduction in the number of calcium oxalate crystals (Figs. 1c, 1d) in medulla region after 15 days treatment, as equiefficacious to cystone treatment at 500 mg/kg dose level (Fig. 1e).

Table 3:

Groups	Serum Total Cholesterol (mg/dl)	Triglycerides (mg/dl)
I	77.95 ± 0.98 ^b	77.4 ± 1.45 ^b
II	116.7 ± 1.5	100.2 ± 1.38
III	92.55 ± 2.09 ^b	94.4 ± 2.74
IV	109.9 ± 1.69 ^a	98.4 ± 1.50
V	91.37 ± 2.16 ^b	92.5 ± 1.63 ^a

Group I (Sham control), Group II (Urolithiatic Control), Group III (Cystone 500 mg/kg), Group IV (CALHAE 300 mg/kg), Group V (CALHAE 600 mg/kg).

^ap<0.05, ^bp<0.01 One way ANOVA followed by Dunnett's posthoc test (n=6) as compared with group II.

Discussion

In the present study male rats were chosen because they bear a resemblance to human urinary system [17] and some earlier studies

Table 4:

Groups	LDL (mg/dl)	HDL (mg/dl)	TBARS (nM/mg)
I	16.6 ± 0.48 ^b	42.62 ± 1.54 ^b	--
II	25.08 ± 0.73	32.08 ± 1.70	291.02 ± 13.8
III	22.45 ± 0.86	34.12 ± 1.32	174.6 ± 13.01 ^b
IV	24.33 ± 1.60	31.53 ± 0.93	189.7 ± 14.3 ^b
V	24.05 ± 0.58	33.32 ± 1.18	153.5 ± 10.8 ^b

Group I (Sham control), Group II (Urolithiatic Control), Group III (Cystone 500 mg/kg), Group IV (CALHAE 300 mg/kg), Group V (CALHAE 600 mg/kg). TBARS: Thiobarbituric acid reactive substance.

have also shown that the amount of stone deposition in female rats was significantly less [18]. Long-term administration of ethylene glycol induced urolithiasis and related oxidative changes in these rats.

The interesting finding of our study was the observation of high concentration of total cholesterol, triglycerides and LDL levels in serum of ethylene glycol induced urolithiatic rats. Administration of CALHAE significantly reduced the cholesterol and triglyceride levels in serum whereas both the doses did not show reduction in LDL levels and improvement in HDL levels. Moreover elevated serum cholesterol and triglycerides levels did not cause glucose intolerance in urolithiatic rats, which was confirmed by oral glucose tolerance test (OGTT). From the histopathological studies, it was clearly observed that vehicle treated rat kidney tissue was devoid of calcium oxalate crystals. In contrast ethylene glycol induced urolithiatic rat kidney tissues shown the deposition of calcium oxalate crystals in medulla region and in renal tubules, which are large polyclonal crystals. The urolithiatic rats treated with CALHAE have shown reduction in the number of calcium oxalate crystals in medulla region after 15 days treatment, as equiefficacious to cystone treatment. The serum lipid profile is generally considered as a reflection of the tissue metabolism and the permeability of cell membrane to various ions, which in turn depends on lipid composition [19]. It has been reported that hyperlipidemia is secondary cause to nephrolithiasis, and it can aggravate the primary renal disorder [20]. Hyperlipidemia has an adverse effect on glomerular function on normal and experimental animals [21]. Serum total

cholesterol was raised in the stone forming rats [22].

The abundance of polyunsaturated fatty acids (PUFA) makes the kidney an organ particularly vulnerable to reactive oxygen species (ROS) attack [23]. ROS causes hyperlipoproteinemia and lipoprotein nephropathy through fibrosis, inflammation, Lipid peroxidation, and antioxidant enzyme modulation [24]. Oxidative stress may be caused by increased concentrations of free oxalate ions or by insoluble calcium oxalate interacting directly with renal epithelial cells, or may originate from other inflammatory events. Exposure to high concentrations of oxalate can induce oxidative stress, as shown by increased lipid peroxidation [25]. In the current study, increased TBARS levels in the ethylene glycol induced urolithiatic rats were observed and treatment with CALHAE and cystone has reduced the TBARS levels.

Results from our in vitro and in vivo studies demonstrated that exposure to high lipid levels results in greater production of ROS, leading to lipid peroxidation and the inhibitory effect of CALHAE on calcium oxalate crystals in the kidney.

Conclusion

In the present study administration of the CALHAE has reduced the growth of kidney stones in ethylene glycol induced urolithiatic rats, supporting folk information regarding antiurolithiatic activity of *C.aromaticus*. Anti hyperlipidemic and potent antioxidant properties of *C.aromaticus* could be of great significance in diminution of calcium oxalate crystals formation in the kidney. Further long-term studies are warranted to clarify the mechanism and

C.aromaticus can play a vital role in urolithiasis therapy.

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