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Effect of ethanolic extract of leaves of *Cocculus hirsutus* (L.) diels on experimentally induced urothiliasis in rats

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Abstract

In the present study, we report anti-urolithiasis effect of ethanolic extract of leaves of *Cocculus hirsutus* (L.) Diels (CH-eth) on the biochemical and histopathological parameters against sodium oxalate (NaOx) induced urolithiasis model in rat. The increased severity of microscopic calcium oxalate (CaOx) crystal deposition along with increased CaOx concentration in kidney was seen after seven days of NaOx (70 mg/kg, i.p) pretreatment. CH-eth (100 and 200 mg/kg, p.o.) and standard marketed formulation, Cystone® (500 mg/kg, p.o.) caused significant reversal of NaOx induced changes in ion excretion and urinary CaOx concentration in seven day treatement (prophylactic and therapeutic schedule). In conclusion, CH-eth showed beneficial effect against urolithiasis by decreasing CaOx excretion and preventing crystal deposition in kidney tubules.

Key words: Calcium oxalate, *Cocculus hirsutus*, urolithiasis, nephrolithiasis, Sodium oxalate.

1. Introduction

Urolithiasis is the third most common disorder of the urinary tract. The world wide incidence of urolithiasis is quite high and in north India more than 80 % of urinary calculi are calcium oxalate stones alone or calcium oxalate mixed with calcium phosphate. In spite of many advances in the field of medicine, options seems to be limited for the treatment of renal calculi or hyperoxaluria found in many stone formers [1, 2]. Hyperoxaluria is the main initiating factor of human idiopathic calcium oxalate (CaOx) stone disease/ Oxalate is a powerful crystallization-driving factor present in urine, retention of which enhances cell injury and causes early stages of lithogenesis [3, 4]. It is evident that depending on the cause,

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different (sympotomatic, prophylactic, therapeutic) therapies are appropriate [1]. Phytomedicines have offered an alternative source of therapy for many diseases including urolithiasis [5-7].

Cocculus hirsutus (Menispermaceae) is a widely growing plant found in the plains of India, in dry localities and is used medicinally by the Indian tribes for a wide range of ailments [8, 9]. The aqueous extract of the aerial parts and the roots are used for the treatment of rheumatism, fever and also as laxative [10]. Roots of *Cocculus hirsutus* have been mentioned in the traditional literature as bitter, acrid, alternative, laxative, demulcent, antiperiodic in fever, tonic and diuretic [10]. The juice of leaves coagulates in water and forms mucilage, which is used externally as a cooling and soothing application in prurigo, eczema and impetigo [11].

While *C. hirsutus* is used medicinally by the Indian tribes for kidney problems [8, 9], it is also been reported to have diuretic activity [10] and shown to be efficacious for prevention of calcium-related renal stone formation [12-15] Therefore, the present study was undertaken with the objective to investigate the effect of ethanolic extract of leaves of *Cocculus hirsutus* (CH-eth) on the biochemical and histopathological parameters of NaOx induced urolithiasis model in rat.

2. Materials and Methods

2.1. Plant material and preparation of extract

The plant of *Cocculus hirsutus* (locally called as Vasanvel / Tana) was collected from Katraj Ghat, Pune and authenticated at Agharkar Research Institute, Pune by expert taxonomist (Dr. A.M. Mujumdar, Head, Botany Department) and voucher specimen was deposited at that Institute (Specimen Number WP–021). The leaves were separated from the plant, dried in shade and powdered in grinder. The powder of leaves was extracted successively with petroleum ether (60°C-80°C) and absolute alcohol (ethanol) using Soxhlet apparatus. The extracts were dried at room temperature. The dried ethanolic extract was weighed (Yield -4.29 %) and used for study purpose.

2.2. Chemicals and apparatus

Creatinine and uric acid estimation kit (Accurex Biomedical Pvt Ltd., Mumbai, India), Zinc wire and sodium oxalate (S.D. Fine Chem. Ltd, Mumbai), and anesthetic ether (TKM Pharma, Hyderabad, India) were purchased and used for the study. Cystone® (Himalaya Herbal Healthcare, Bangalore, India) was used as a standard drug. The detail composition and its urolithiatic action of Cystone® formulation is well documented [2]. Cystone®, contains extracts of Shilapushpa (*Didymocarpus pedicellata*) 130 mg, Pashanbheda (*Saxifraga ligulata*) 98 mg, Manjishtha (*Rubi cordifolia*) 32 mg, Nagarmustha (*Cyperis scariasus*) 32 mg, Apamarga (*Achyranthes aspera*) 32 mg, Gojiha (*Onosoma bracteatum*) 32 mg, Sahadevi (*Vernonia cinereas* 32 mg, Hajrul yahood bhasma (*limesilicate calyx*) 32 mg and Shilajeet (Mineral pitch) 26 mg.

2.3. Animals

Male Wistar rats (150-250 g.) were purchased from National Toxicology Centre, Pune, India. Animals were housed under standard condition of temperature (24 ± 2°C) and relative humidity of 30-70% with a 12h:12h light: dark cycles. The animals were fed with standard pellet diet (Chakan Oil mills, Sangli) and provided water ad libitum. All the experiments were carried out between 9.00 and 16.00 h. The protocol was approved by Institutional Animal Ethics Committee (IAEC).

2.4. Phytochemical Studies

Standard phytochemical screening tests were carried out for various constituents of the

aqueous extract of leaves of *Cocculus hirsutus* according to the methods of Trease and Evans [16]. The presence or absence of various phytoconstituents like saponins, carbohydrates, alkaloids, flavonoids, and phenolic compounds were observed by preliminary phytochemical screening.

2.5. Acute Oral Toxicity Study

Male Swiss albino mice weighing 18-22 g were dosed with *Cocculus hirsutus* extract and were observed for any symptoms of toxicity for 48 hours as per OECD guidelines 425 and LD50 was estimated using AOT 425 software (Westat, EPA, USA). Based on the results obtained from this study, the doses for further pharmacological studies were fixed to be 100, and 200 mg/kg, p.o.

2.6. Anti-urolithiasis activity against sodium oxalate (NaOx) induced urolithiasis

The effect of CH-eth was measured against NaOx induced urolithiasis [17]. All rats were housed in metabolic cages individually for entire duration of the experiment. Separate group of rats were used to study the prophylactic and therapeutic effects of treatments. In prophylactic schedule, CH-eth (100 and 200 mg/kg, p.o.), Cystone® (500 mg/kg, p.o.), or vehicle (0.5% CMC) were administered with NaOx (70 mg/kg, i.p.) for seven days to separate group of 6 animals each. In the separate experiment (therapeutic schedule), urolithiasis was allowed to develop by NaOx (70 mg/kg, i.p.) administration for first seven days of the experiment.

The treatments (CH-eth, Cystone® or vehicle) were given from day-8 to 14. On 7th day (prophylactic) and 14th day (therapeutic), the urine was collected (6 hrs after treatments) and thymol was added as a preservative. Urine samples were then analyzed for creatinine and uric acid concentration (Auto analyzer, Secomam), sodium, potassium, and chloride ion excretion (electrolyte analyzer, Biolyte 2000, Taiwan) and pH (pH meter, model EQ-614). On 7th and 14th day, the animals were sacrificed. Their kidneys were harvested. One kidney of each animal was processed for histopathology and another kidney was used to determine CaOx deposition. [18]. Urine and kidney calcium oxalate concentrations were determined by method of Hodgkinson and Williams [17].

2.7. Statistical analysis

The results are expressed as Mean ± SEM. Comparison between the groups was made by analysis of variance (ANOVA) followed by Dunnett's test.

3. Results

On gross examination, all animals were devoid of toxic symptoms and mortality when CH-eth was given in doses upto 2000 mg/kg, p.o.

3.1. Urine output and excretion of constituents

Administration of NaOx (70 mg/kg, i.p) did not cause any change in urinary output, pH of urine, uric acid and creatinine excretion after seven days of treatment as compared with vehicle treated group. However, significant ($P < 0.001$) reduction in serum sodium and chloride ion excretion and significant increase ($P < 0.001$) in serum potassium ions and calcium oxalate excretion were observed (Table 1). CaOx levels in kidney samples were also significantly elevated ($P < 0.001$). These results indicated induction of calcium oxalate urolithiasis in rat kidneys.

Administration of CH-eth (100 and 200 mg/kg, p.o.) and Cystone® (500 mg/kg, p.o.) caused significant ($P<0.01$) reversal of NaOx induced changes in ion excretion and urinary CaOx concentration after 7 days of treatment in prophylactic schedule (Table 1).

Table 1. Effect of ethanolic extract of Cocculus hirsutus leaves on urine parameters against NaOx induced urolithiaisis

Parameter	Schedule	Vehicle	NaOx	CH-eth (100)	CH-eth (200)	Cystone®
Urine output	PR	3.05 ± 0.08	2.33 ± 0.08	4.07 ± 0.2	4.28 ± 0.24	6.43 ± 0.08
(mL.)	TH	2.88 ± 0.16	2.07 ± 0.08	3.18 ± 0.12	3.05 ± 0.08	4.98 ± 0.08
pH	PR	7.45 ± 0.13	6.23 ± 0.012	7.22 ± 0.06	7.32 ± 0.07	7.98 ± 0.16
	TH	7.53 ± 0.21	6.53 ± 0.017	6.72 ± 0.12	6.55 ± 0.17	6.78 ± 0.13
Sodium	PR	159.50 ± 2.26	100.67 ± 5.27$^{\#}$	8.67 ± 3.56**	142.87 ± 2.26***	235.17 ± 6.56***
(mEq./L.)	TH	158.00 ± 2.48	100.67 ± 3.13$^{\#}$	105.17 ± 3.76	123.33 ± 3.23***	174.17 ± 4.7***
Potassium	PR	219.83 ± 3.61	254.67 ± 36.19$^{\#}$	272.17 ± 4.31**	270.00 ± 4.72**	298.67 ± 6.14***
(mEq./L.)	TH	215.00 ± 6.71	248.17 ± 5.08$^{\#}$	232.67 ± 3.17	251.83 ± 2.01	235.50 ± 5.88
Chloride	PR	240.17 ± 2.77	174.50 ± 3.95$^{\#}$	223.33 ± 5.99***	243.00 ± 4.28***	417.83 ± 6.24***
(mEq./L.)	TH	257.33 ± 7.68	191.00 ± 4.08$^{\#}$	207.33 ± 7.03*	214.50 ± 7.65***	340.83 ± 6.15***
Uric acid	PR	5.12 ± 0.32	5.88 ± 0.19	3.18 ± 0.16	3.50 ± 0.23	3.02 ± 0.11
(mg/dL)	TH	6.62 ± 0.44	5.12 ± 0.21	6.10 ± 0.0	4.93 ± 0.27	4.68 ± 0.15
CaOx	PR	20.32 ± 1.43	141.38 ± 3.19*	117.25 ± 4.01***	99.69 ± 3.65***	84.38 ± 2.72***
(mg/dL)	TH	20.87 ± 2.23	161.98 ± 6.36$^{\#}$	119.32 ± 4.9***	120.60 ± 5.07***	99.69 ± 3.65***
Creatinine	PR	0.59 ± 0.04	0.20 ± 0.01	0.58 ± 0.06	0.62 ± 0.07	0.72 ± 0.07
(mg/dL)	TH	0.56 ± 0.07	0.81 ± 0.01	0.39 ± 0.02	0.29 ± 0.02	0.56 ± 0.06
CaOx (kidney)	PR	61.08 ± 2.51	222.77 ± 5.23$^{\#}$	174.38 ± 5.88***	154.40 ± 5.97***	83.25 ± 2.17***
(mg/dL)	TH	58.72 ± 3.94	236.30 ± 8.69$^{\#}$	183.00 ± 5.38***	167.95 ± 6.95***	92.19 ± 2.96***

Treatments are given for 7 days, PR - Prophylactic schedule, TH - Therapeutic Schedule,
Values are expressed as mean ± S.E.M., n = 6, ### P < 0.001, as compared with Vehcle treated group, * P < 0.05, ** P , 0.01 compared to NaOx treated group, using tw-way ANOVA followed by post hoc dunnett's test.

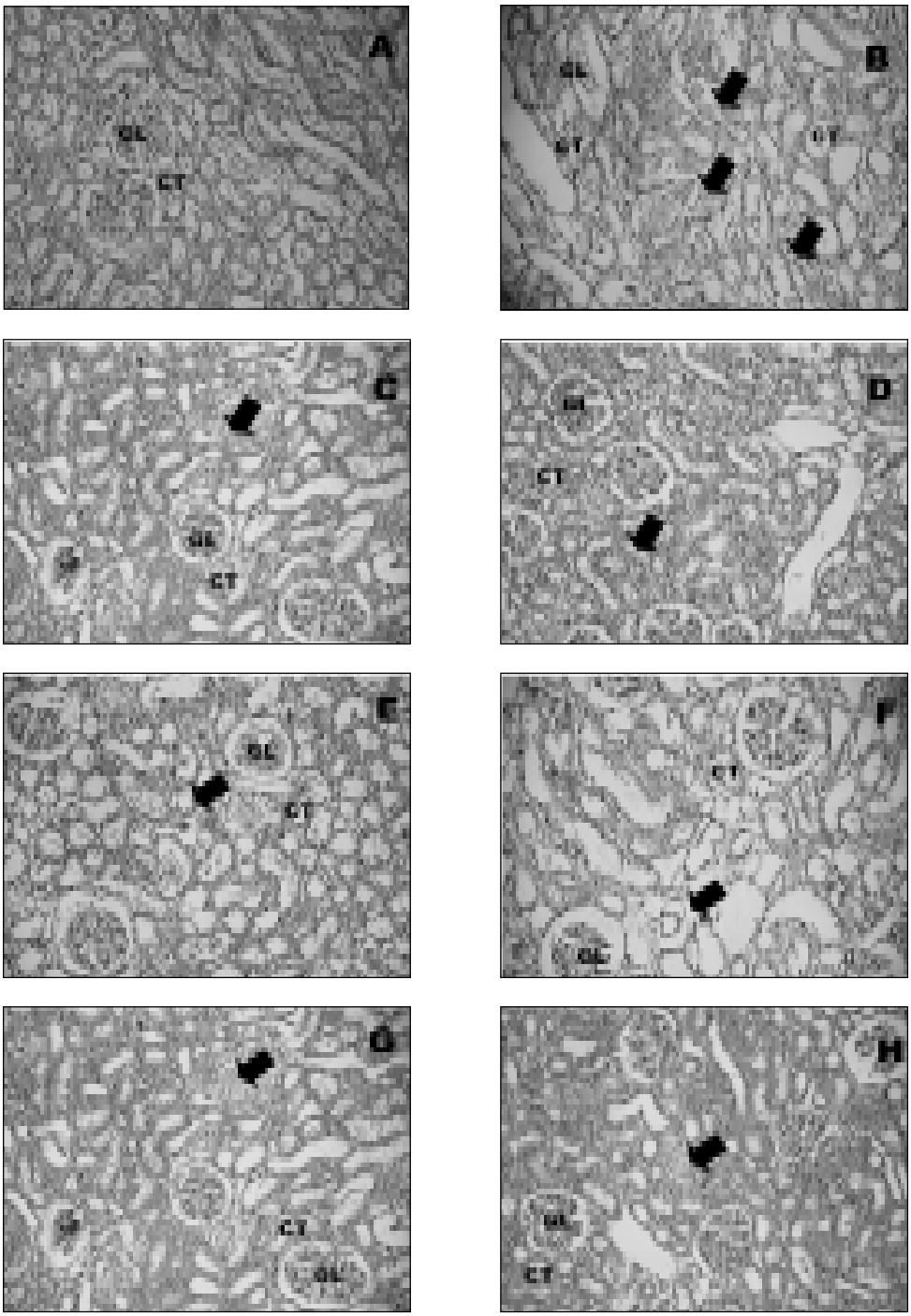

Fig. 1:
Photomicrographs of kidney sections of rats treated with vehicle, Cystone®, and ethanolic extract of *Cocculus hirsutus* leaves (CH-eth) for 7 days against NaOx induced urolithiasis. (A) Vehicle (B) NaOx (70 mg/kg, i.p., 7 days) (C)) NaOx + Cystone® (500 mg/kg, p.o., PR) (D)) NaOx+Cystone® (500 mg/kg, p.o., TH) (E) NaOx+Ch-eth (100 mg/kg, PR) (F) NaOx+CH-eth (200 mg/kg , TH) (G) NaOx+CH-eth (100 mg/kg, TH) (H) NaOx+CH-eth (200 mg/kg; TH). Arrows indicate CaOx crystal deposition. CT-collecting tubule, GL – Glomerulus, PR-Prophylactic, TH-therapeutic, Magnification 100x

However, in therapeutic schedule, improvements in NaOx induced urolithiasis in terms of sodium and potassium excretion were obtained only at dose of 200 mg/kg. At lower dose (100 mg/kg, p.o.), CH-eth reversed reduction of chloride excretion but unable to reverse NaOx induced sodium and potassium excretion. On the other hand, therapeutic administration of Cystone® revered NaOx induced urolithiasis changes in terms of ion (sodium and chloride) and CaOx excretion except potassium ion excretion, which remained unaffected.

3.2. Histopathological evaluation of rat kidney

The photomicrograph of sections of rat kidneys treated with NaOx (Figure 1B) showed moderate to maximum crystals deposition with focal acute tubular necrosis, along with dilated collecting tubules and focal tubular atrophy in renal tubules and mild infiltration of interstitium mononuclear cells as compared with vehicle treated rats (Figure 1A).

Cystone® (500 mg/kg, p.o) and CH-eth (100 mg/kg, p.o) treatments caused mild crystal deposition with fragmentation in both prophylactic and therapeutic schedule (Figure 1C, D, E and F). CH-eth at 200 mg/kg, p.o dose showed mild CaOx crystal deposition with dilated collecting tubules after prophylactic treatment (Figure 1G) and maximum decrease in CaOx crystal deposition with normal collecting tubules after therapeutic treatment (Figure 1H). The blood vessels and glomeruli showed normal structure in all the kidney sections (Figure 1).

4. Discussion

In spite of advances in the understanding of urolithogenesis, there is lack of satisfactory drug treatment of the 'idiopathic' oxalocalcic stone-formers (or hyperoxaluria or hypercalciuria). This might be due to many causes that provoke the disease in a non-uniform group of patients. Thus, the genesis of the calculus is attributed to a deficit of crystallization inhibitors (nucleation inhibitors) and/or an increase of promoters (heterogeneous nucleation) [19].

A number of animal models have been used for the study of nephrolithiasis [20-22]. NaOx (70 mg/kg, i.p. for seven days) induced hyperoxaluria model in rats was used in the present study because of close resem blance of rat urinary system to that of the humans [23].

Both histopathological study for CaOx crystal deposition in kidney sections and biochemical assay for determination of CaOx levels in kidney and urine were carried out during the study. Experimental induction of hyperoxaluria results in rapid formation of CaOx crystals in renal tubules of experimental animals. These are evident from our results of NaOx treated group where significant elevation in calcium oxalate excretion and CaOx levels in kidneys (Table 1) were observed. These results are in line with the clinical reports of CaOx urolithiasis patients [24, 25].

The appearance of CaOx in renal tubules following NaOx injection is associated with necrosis of tubular cells, which results in exposure of tubular basal lamina and formation of luminal cellular debris. The calcium oxalate crystals do cause cytolysis of polymorphonuclear leukocytes following phagocytosis and may be destructive to renal epithelium [18, 26].). NaOx challenge brings about rapid increase in urinary excretion of CaOx and formation of crystals takes place. These crystals deposit progressively in the cortex, medulla and renal tubules [26]. In the present study, the increased severity of microscopic kidney crystal deposition after seven day treatment with NaOx (Figure 1B) correlated well with increased calculi oxalate concentration in kidney (Table 1). The treatment of CH-eth and Cystone® caused significant reduction in CaOx excretion and CaOx in the kidney (Table 1) suggested their beneficial effects against CaOx deposition in urolithiasis.

The treatment of CH-eth and Cystone® increased urinary excretion of sodium and chloride (Table 1). Elevations in levels of sodium and chloride excretion have beneficial effect in preventing calculi formation due to super saturation of these lithogenic substances. The relationship between diuretic potential and anti-urolithasis effect is well documented [7, 27]. Diuresis promoted the stones evacuation from the ureter [28] and thaizide diuretics are clinically used against urolithiasis patients [13, 29]. Furthermore, arieal parts of *C. hirsutus* was reported earlier to have diuretic activity [30] which may contribute to its urolithiatisis activity.

The antiurolithiasis effect of CH-eth can be attributed to the presence of phytochemicals such as alkaloids, phenolic compounds, flavonoids, and glycosides that were detected in our preliminary phytochemical studies. These results also commensurate with earlier reported phytochemicals of *Cocculus hirsutus* i.e. essential oil, ß-sitosterol, ginnol, glycosides, sterols and alkaloids [31, 32].

Active involvement of free radical-mediated lipid peroxidation-induced membrane damage was found in the pathogenesis of calcium oxalate crystal deposition and retention [33]. Furthermore, the inhibitory effect of flavonoids against calcium oxalate urolithiasis [34] and beneficial effects of lupeol against hyperoxaluria [35, 36] is also reported. Also usefulness of antioxidant potential for the prophylactic option against renal cell injury associated kidney stone formation is demonstrated recently [37]. Therefore, presence of phenolic compounds (especially flavonoids) by virtue of their antioxidant potential are suggested to play important role in anti-urolithiasis effects of *C. hirsutus* leaves. Some triterpenoid glycosides were reported to inhibit the formation of CaOx stones in rat kidneys by increasing the output of urine, decreasing the excretion of calcium and increasing the urinary excretion of citrate [38] and also possess antioxidant potential [39]. Presence of triterpenoids glycosides in CH-eth was also suggested to play significant role in its anti-urolithiasis effect. Further studies for identification of the active constituents for anti-urolithisis effect of the leaves of *Cocculus hirsutus* are required.

In conclusion, CH-eth showed beneficial effect against urolithiasis by decreasing CaOx excretion and preventing crystal deposition in kidney tubules.

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