

Antilithiatic Effect of Ethanolic Extract of *Stevia Rebaudiana Bert*

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Summary

The aim of the present study was to investigate the effect of oral administration of ethanolic extract of *Stevia rebaudiana* Bertoni (Asteraceae) leaves against ethylene glycol-induced nephrolithiasis in albino rats. Lithiasis was induced in rats by administrating 0.75 % v/v ethylene glycol in drinking water for 28 days and was manifested by hyperoxaluria as well as increased renal excretion of calcium, phosphate and low urinary magnesium content. Supplementation with ethanolic extract (100 mg/kg b.w., p.o.) of *S. rebaudiana* leaves significantly reduced the elevated urinary oxalate, showing a regulatory action on endogenous oxalate synthesis.

The increased deposition of stone forming constituents in the kidneys of calculogenic rats was significantly lowered by curative and preventive treatment using ethanolic extract (100 mg/kg b.w., p.o.) of *S. rebaudiana* leaves. From this study, we conclude that both the prophylactic and therapeutic treatment with ethanolic extract of leaves of *S. rebaudiana* had an inhibitory effect on crystal growth, with improvement of kidney function as well as cytoprotective effect.

Keywords: *Stevia rebaudiana*, ethylene glycol, creatinine, antiurolithiatic activity.

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Introduction

Urinary stone disease has afflicted humankind since antiquity and can persist, with serious medical consequences, throughout a patient's lifetime. In addition, the incidence of kidney stones has been increased in western societies in the last five decades, in association with economic development. Most calculi in the urinary system arise from a common component of urine, e.g. calcium oxalate (CaOx), representing up to 80% of analyzed stones[1].

A large number of plant drugs have been used in India since ancient times which claim efficient cure of urinary stones. Amongst the medicinal plants used in urolithiasis are 'patharphor' (*Didymocarpus pedicellata*), several *Bergenia* species, three species of *Tribulus* (*T. systoides*, *T. terrestris* and *T. alatus*), 'manjit' (*Rubia cordifolia* and *Rubia tinctorum*), 'varuna' (*Crataeva nurvala*) and 'imli' (*Tamarindus indica*)[2].

Stevia rebaudiana (Bertoni) family Asteraceae is an herbaceous perennial plant indigenous to Paraguay and Brazil where its leaves are used by the local Guarani Indians as natural sweetener for hundreds of years. About 150 stevia species are known, among them *S. rebaudiana* is the only one with significant sweet tasting properties. This plant is of world wide importance today because its leaves are used as non-nutritive high potency sweetener primarily in Japan, Korea, China and South America. The consumption of stevia extract in Japan and Korea is about 200 and 115 tons/year, respectively [3]. The water extract of *S. rebaudiana* has beneficial effects on human health, including hypoglycemic [4], hypotensive [5] and renal effects [6]. Its leaves contain nine sweet glycosides. They possess an ent-kaurene diterpene steviol skeleton (ent-13-hydroxy kaur-16-en-19-oic acid). Generally dominant are stevioside (6–10%), rebaudioside-A (2–4%) while other minor glycosides are present up to 1–2% in the leaves [7]. Many analytical methods have earlier been reported in literature for the separation and quantification of diterpene glycosides from the leaves of *S. rebaudiana*.

Based on the above facts and in continuation of our research work on *S. rebaudiana* we report herein the antiurolithiatic activity of various extracts of *S. rebaudiana*.

Materials and Methods

Plant material

The leaves of *S. rebaudiana* were collected in March 2009 from herbal garden of, Department of Pharmacy Bhimtal District, Nainital, Uttarakhand India. Further taxonomic identification was conducted by Dr. K N Pandey, Head, Department of Botany, Kumaun University, Nainital, Uttarakhand, India. A voucher specimen was deposited in the herbarium of our laboratory under the number (Pharm/0102/09)

Preparation of the extract

The air-dried leaves of *S. rebaudiana* (50 g) were powdered and then extracted with 500 ml of ethanol by using soxhlet apparatus. The crude extract was filtered and evaporated under reduced pressure to give a viscous dark mass with a percentage yield of 4.5% (w/w). This crude extract was dissolved in water or solvent and used for the assessment of antiurolithiatic activity.

Pharmacological screening for antiurolithiatic activity

Animal Selection: For acute toxicity studies, albino mice of either sex weighing between 25 and 30 g were selected and healthy adult male albino rats weighing between 150 and 200 g were selected for the antiurolithiatic activity. The animals were acclimatized to standard laboratory conditions (temperature: 25 ± 2 °C) and maintained on 12-h light: 12-h dark cycle. They were provided with regular rat chow (Ashirwad Industries, Chandigarh, India) and drinking water *ad libitum*. The animal care and experimental protocols were in accordance with Institutional Animal Ethical Committee (IAEC).

Acute Toxicity Studies: The acute oral toxicity study was carried out as per the guidelines set by Organization for Economic Co-operation and Development (OECD) received from Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA). One-tenth of the median lethal dose (LD_{50}) was taken as an effective dose [8]

Ethylene glycol induced urolithiasis model: Ethylene glycol induced hyperoxaluria model[9] was used to assess the antilithiatic activity in albino rats. Animals were divided into seven groups containing six animals in each. Group I served as control and received regular rat food and drinking water *ad libitum*. Ethylene glycol (0.75%) in drinking water was fed to Groups II to VII for induction of renal calculi till 28th day. Group III received standard antiurolithiatic drug, cystone (750 mg/kg body weight) from 15th day till 28th day. Groups IV and group V served as curative regimen (CR). Group IV and group V received ethanolic extract (100 mg/kg and 200 mg/kg, respectively) from 15th day till 28th day. Group VI and group VII received ethanolic extract (100 mg/kg and 200 mg/kg, respectively) from 1st day till 28th day and served as preventive regimen (PR). All extracts were given once daily by oral route.

Assessment of antiurolithiatic activity

Collection and analysis of urine: All animals were kept in individual metabolic cages and urine samples of 24-h was collected on 28th day. Animals had free access to drinking water during the urine collection period. A drop of concentrated hydrochloric acid was added to the urine before being stored at 4 °C. Urine for calcium[10], phosphate[11] and oxalate[12]content were analyzed.

Serum analysis: After the experimental period, blood was collected from the retro-orbital under anaesthetic conditions and animals were sacrificed by cervical decapitation. Serum was separated by centrifugation at 10,000 x g for 10 min and analyzed for creatinine, urea nitrogen[13] and uric acid[14].

Kidney homogenate analysis: The abdomen was cut open to remove both kidneys from each animal. Isolated kidneys were cleaned off extraneous tissue and preserved in 10% neutral formalin. The kidneys were dried at 80 °C in a hot air oven. A sample of 100 mg of the dried kidney was boiled in 10 ml of 1 N hydrochloric acid for 30 min and homogenized. The homogenate was centrifuged at 2,000 x g for 10 min and the supernatant was separated[15]. The calcium ^[10], phosphate ^[11] and oxalate ^[12] content in kidney homogenate were determined.

Statistical analysis

Results were expressed as mean ± SEM. Differences among data were statistically analyzed using one-way ANOVA followed by Student Newman Keul's test (Graphpad Prism software for Windows, Version 2007). The statistical significance was set accordingly[16].

Results

From acute toxicity studies, it was observed that extracts were found to be safe upto a maximum dose of 2000 mg/kg b. w. except a few changes in the behavioral response like alertness, touch response and restlessness. The dose 1/10th (200mg/kg) of the maximum tolerated dose 2000 mg/kg b.w. was chosen for further studies.

In the present study, hyperoxaluria was induced by chronic administration of 0.75% (v/v) ethylene glycol aqueous solution to Wistar rats. Oxalate, calcium and phosphate excretion were grossly increased in calculi-induced animals (Table 1, group II). Cystone treatment significantly lowered the elevated levels of oxalate, calcium and phosphate in urine and kidney (Table, group III). Ethanolic extract of *S. rebaudiana* leaves (100 mg/kg and 200 mg/kg) significantly lowered the elevated levels of oxalate, calcium and phosphate in urine and kidney in curative regimen (CR) and preventive regimen (PR) as compared to calculi treated animals (Table 1, group IV,V,VI,VII). The deposition of the crystalline components in the renal tissue, namely oxalate, phosphate and calcium, were increased in the calculi-induced animals (Table 1, group II). The ethanolic extract of *S. rebaudiana* leaves treatment significantly reduced the renal content of these stone forming constituents in both regimens (Table 1, groups IV to VII). The serum uric acid, creatinine and BUN were significantly increased in calculi-induced animals (Table 1, group II) as compared to control, while serum creatinine was elevated in group II, indicating marked renal damage. However, *S. rebaudiana* leaves extract treatment is curative (Table1, group IV and V) and preventive (Table 1, group VII and VII) and significantly lowered the elevated serum levels of creatinine, uric acid and BUN.

Table 1: Effect of *S. rebaudiana* leaves on urinary and serum parameters in experimental mice

Animals	Group I	Group II	Group III	Curative regime		Preventive regimen	
Parameter (unit)	Normal	Calculi-induced	Cystone-treated	(CR-I and CR-II)		(PR-I and PR-II)	
	(CI)	(CT)		Group IV	Group V	Group VI	Group VII
Urine (mg/dl)							
Oxalate	0.32±0.03	3.74±0.12 ^a	0.52±0.04 ^b	1.28±0.04 ^c	0.85±0.04 ^b	1.06±0.08 ^c	0.75±0.03 ^b
Calcium	1.24±0.06	4.39±0.10 ^a	1.53±0.05 ^b	1.87±0.07 ^c	1.76±0.13 ^b	1.86±0.07 ^c	1.69±0.07 ^b
Phosphate	3.72±0.04	7.48±0.07 ^a	3.41±0.10 ^b	4.14±0.10 ^c	4.04±0.07 ^b	4.12±0.09 ^c	3.85±0.05 ^b
Kidney							
Oxalate	3.72±0.05	5.29±0.05 ^a	1.63±0.06 ^b	2.31±0.06 ^c	1.83±0.03 ^b	2.07±0.08 ^c	1.76±0.05 ^b
Calcium	3.20±0.04	4.79±0.17 ^a	3.41±0.07 ^b	4.10±0.04 ^c	3.65±0.07 ^b	3.45±0.07 ^c	3.01±0.06 ^b
Phosphate	2.36±0.03	3.82±0.10 ^a	2.51±0.03 ^b	2.81±0.10 ^c	2.21±0.06 ^b	2.75±0.06 ^c	2.51±0.06 ^b
Serum (mg/dl)							
BUN	38.21±0.14	51.21±0.50 ^a	39.61±0.45 ^b	42.81±0.37 ^c	42.81±0.31 ^b	41.70±0.01 ^c	40.07±0.39 ^b
Creatinine	0.72±0.01	0.96±0.03 ^a	0.81±0.01 ^b	0.91±0.01 ^c	0.81±0.02 ^b	0.8±0.20 ^c	0.75±0.01 ^b
Uric acid	1.51±0.06	3.64±0.12 ^a	1.70±0.04 ^b	2.14±0.07 ^c	1.81±0.06 ^b	2.12±0.05 ^c	1.89±0.04 ^b

Group I represents, normal animal group. Group II represents, calculi-induced animals. Group III represent cystone treated group. Group IV and VI represents, administration of *S. rebaudiana* (100 mg/kg) extract calculi-induced animals. Group V and VII represents, administration of *S. rebaudiana* (200 mg/kg) extract calculi-induced animals. Values are expressed as ± SEM. a=p<0.001 Vs normal group. b=p<0.001 Vs calculi-induced group. c=p<0.05 Vs calculi-induced group.

Discussion

Chronic administration of 0.75% ethylene glycol aqueous solution to Wistar rats resulted in hyperoxaluria. Urinary stone formation is mainly the result of supersaturation of urine with certain urinary salts such as calcium oxalate, the most common constituent of kidney stones [17]. Oxalate and calcium excretion in urine were grossly increased in calculi-induced animals (Table 1, group II). The enzymatic disturbances are the causative factors for the idiopathic hyperoxaluria; while, increased urinary oxalate concentration is due to defective intestinal absorption of oxalate, which plays a vital role in enteric hyperoxaluria [18].

Hyperoxaluria is far more significant risk factor in the pathogenesis of renal stones than hypercalciuria, the changes in urinary oxalate levels are relatively much more important than those of calcium. The increase in calcium deposition in kidney and its urinary excretion results in defective renal tubular reabsorption or an increase in its absorption from the intestine. Ethanolic extract of *S. rebaudiana* leaves lowered the levels of oxalate and calcium in urine and kidney.

In the present study a remarkable increase in urinary phosphate was observed in calculi-induced rats. Increased urinary phosphate excretion along with oxalate stress seems to provide an environment appropriate for stone formation by forming calcium phosphate crystals, which epitaxially induces calcium oxalate deposition [19].

Treatment with Ethanolic extract of *S. rebaudiana* leaves restores normal urinary phosphate level; thereby stone formation risk is reduced. 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 such as urea, creatinine and uric acid get accumulated in blood. In calculi induced rats (Table 1, group II), the elevated serum levels of creatinine, uric acid and BUN indicate marked renal damage. Since *S. rebaudiana* leaves are reported to have diuretic activity [20] this may be beneficial in the management of urolithiasis.

The present study shows that the administration of *S. rebaudiana* effectively prevented the development of urolithiasis in rats. *S. rebaudiana* has a high antioxidant capacity may be due to the presence of flavonoids such as quercetin and kaempferol (V. Ani, 2008). Phenolic compounds present in *S. rebaudiana* may prevent the lipid peroxidation induced renal damage caused by calcium oxalate crystal deposition in the kidney. Hence, *S. rebaudiana* can prevent calcium oxalate crystal attachment as well as stone formation. Other possible mode of action includes excessive excretion or decreases the concentration of salts in the urine, which leads to prevent the supersaturation of the crystallizing salt. The significant lowering of serum levels of accumulated waste products is attributed to the enhanced glomerular filtration rate produced by *S. rebaudiana* as reported and it also hastens the process of dissolving the preformed stones in CR and prevention of new stone formation in urinary system on prophylactic treatment.

The present investigation showed that the ethanolic extract of *S. rebaudiana* leaves significantly prevents the formation of urolithiasis. This confirms the utility of leaves of *S. rebaudiana* in folklore medicine as remedy for kidney troubles.

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