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Research article

Curative potential of *N. cadamba* methanol fruit extract on experimentally induced urolithiasis in rats

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Key words: Nephrolithiasis, Ethylene glycol, Pizzalto's staining method.

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Abstract

The formation of kidney stone/renal calculi is a complex process including several physicochemical events results in urinary supersaturation and leads to nucleation, growth, aggregation and retention of crystals within the kidneys. Nephrolithiasis is a serious health problem in all societies and worldwide in distribution. Several epidemiological data and clinical studies have reported that calcium oxalate (CaOx) followed by calcium phosphate are the frequently encountered stones in majority of kidney stones. The present study focused on the calcium oxalate induced nephrolithiasis in Wistar albino rats. Five groups of animals were used for the study. Group I, II and III were considered as control received normal drinking water, antilithiatic drug and ethylene glycol respectively for a period of 28 days. Group IV and V were supplemented with the methanol fruit extract of *N. cadmaba* (MFNC) at low dose and high dose after inducing kidney stone. After the experimental period, calcium and phosphorus levels in kidneys and urine, urinary protein, serum creatinine, urea and uric acid in urine sample and microscopic analyses of urine samples were carried out on day 28. Pizzalto's staining methods were used to evaluate the amount and deposition of kidney stones. The results of the study suggested that the extract administered groups showed significant reduction in the stone forming constituents in blood, urine and kidney as compared to the lithiatic groups.

Introduction

Kidney stones are an alarming health problem in all societies throughout the world. It is the process of deposition of minerals in the kidney, ureters and urinary bladder. Even though various medical treatments are used to manage the renal stones, they have revealed limitations and demerits such as residual stone fragments and recurrence rate. Hence the scientific developers generally pointed out to manage the kidney stone with minimal invasive alternatives [1]. Phototherapy is highly esteemed all over the world as a rich source of therapeutic treatment against different diseases. Nature offers the best remedy and is the first pharmacy. The increasing demand on phytotherapy forced the pharmaceutical companies to reconsidered and research on medicinal plants [2]. Multiple mechanism of action of plant remedies is effective in reducing the stone formation and stone deposition. Plant extract exerts their antilithiatic activity by altering the ionic composition of urine like calcium, phosphate, magnesium and citrate. The multiple mechanisms seen in plants to cure lithiasis involve the diuretic activity, crystallization inhibition activity, antimicrobial activity, antioxidant activity, lithotriptic activity, analgesic and anti-inflammatory activity. The aim of the present study is to evaluate the calcium oxalate stone reducing property of the fruits of *Neolamarckia cadamba,* in the wistar albino rats.

Experimental

Materials and methods

Plant material

The fruits of *N. cadamba* were collected from the Botanical Garden, University Campus, Kariavattom ($8^0$37'36N, 76^o50'14E), Thiruvananthapuram and authenticated by the Department of Botany, University of Kerala, Kariavattom (voucher number: KUBH 5811). The methanol extract of the fruit was prepared by the soxhlet extraction method and concentrated using rotary vacuum evaporator under reduced pressure was used for the study. Yield and extractive values were calculated for the methanol fruit extract

Experimental animal model and study protocol

Healthy adult male albino rats of wistar strain weighing between 200- 250g were used for the antinephrolithiatic study. The animals were acclimatized to standard laboratory conditions and maintained at12-hr light and 12-hr dark cycle. Animal experiments were carried out using CPCSEA guidelines (Approval no: IAEC-KU-23/2011-12-ZOOL-GP (3)). The acute oral toxicity study was carried out as per the OECD (Organization for

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Economic Co-operation and Development) guidelines 423. Animals were divided into nine groups containing four rats in each group. Calcium oxalate nephrolithiasis was induced in rats by free access to drinking water containing ethylene glycol (EG) and ammonium chloride (AC). Group 1 served as normal control and received regular standard rat food and drinking water at *ad libitum*. EG and AC in drinking water was given to group II - V for the induction of renal calculi till the 28th day. Group II were treated with the standard anti-urolithiatic drug, cystone (750mg/kg body weight). Dose for the methanol fruit extract was selected as 200 and 400mg/kg body weight. Group III was considered as lithiatic control where as group IV and V received the MFNC at a dose of 200mg/kg and 400mg/kg body weight respectively after treatment with EG+AC in drinking water.

Urine analysis and microscopic examination of urine

During the experimental period, 24hr urine samples were collected on day 28 and were centrifuged and supernatant was analyzed for protein (biuret method), phosphorus (Span Diagnostic Ltd. Surat, Code No. 88LS100-50 using UV- molybdate method) and calcium (Span Diagnostic Limited Surat, Code No.87LS100-60) using O-cresolphthalein-complexone method) and oxalate [3] and the sediment was examined under low and high power objective of a microscope for the detection of abnormal constituents in urine like casts, crystals etc.

Serum analysis

After the experimental period, animals were sacrificed by cervical decapitation and blood was collected by heart puncture under anaesthetic condition. Serum was separated and analyzed for creatinine (alkaline picrate method), urea, blood urea nitrogen (BUN) (Diacetylmonoxime method) and uric acid (Caraway method) [4].

Renal homogenate analysis

The isolated kidneys from the animal groups were cleaned off to remove the extraneous tissues and dried at 80ºC in hot air oven. The homogenate and centrifuged the sample and the supernatant was analyzed for stone forming constituents such as kidney phosphate, calcium, oxalate and protein.

Detection of calcium oxalate crystals

Pizzolato's (1964) [5] staining method was carried out for the detection of calcium oxalate crystals in kidney tissues using silver nitrate and hydrogen peroxide. Calcium oxalate crystals were seen as black in colour.

Statistical analysis

The results were expressed as mean ± SEM. Statistical analysis was performed by one way analysis of variance (ANOVA) followed by Tukey multicomparison test. P values ≤ 0.05 were considered significant.

Results and Discussion

Results

The yield of the fruit extract was found to be 20.26% and alcohol soluble extractive value was 9.21. Urinary protein and phosphorus concentration has significantly ($p < 0.01$) higher in lithiatic control rats. MFNC administration was found to lower these values significantly (Table 1). When compared with the antilithaitic drugs, the significant decrease in the concentration of phosphorus and protein was observed from the groups IV (200mg/kg b. wt) and V (400mg/kg b. wt). Both urine calcium and oxalate concentrations were decreased significantly on 28th day of extract administration in the MFNC treated groups (Table 2). When compared with group I, a significant increase in calcium and oxalate was noticed from group III (lithiatic control group). Serum nitrogenous substances like urea, uric acid and creatinine concentrations were significantly ($P<0.01$) elevated in EG/AC administered groups of rats. However, group IV and V has the potential to decrease the concentration of nitrogenous substances (Table 3). When compared to the control groups (group 1), group III showed significant elevation in kidney phosphorus, calcium and oxalate. When compared with group II, the treatment groups (IV and V) significantly lowered the elevated values (Table 4).

Table 1. Effect of MFNC on urine protein and phosphorus.

Treatment groups	Protein (mg/24hr)	Phosphorus(mg/24hr)	
		14th day	28th day
Group1	3.72±0.20	4.12±1.32	4.76±0.27
Group II	0.15±0.004c**	9.63±3.02	3.79±0.16c**
Group III	23.86±4.25a**b**	10.47±1.2	15.15±1.68a**b**
Group IV	0.05±0.01 c**	5.30±2.1	2.16±0.36c**
Group V	0.79±0.06 c**	3.05±1.6	3.27±0.47c**

Each value is the mean ±SEM for 4 animals, a-indicates significant difference with normal control groups, b- indicates significant difference with cystone treated groups, c-indicates significant difference with lithiatic control groups. *- P<0.05, **-P<0.01.

Table 2. Effect of MFNC on urine calcium and oxalate.

Treatment groups	Calcium (mg/24hr)		Oxalate (mg/24hr)	
	14th day	28th day	14th day	28th day
Group1	0.6±0.14	0.92±0.01	0.58±0.13	0.16±0.03
Group II	1.87±0.34	1.85±0.41	2.69±0.32	1.61±0.09
Group III	2.7±0.54	3.47±0.57 a*	2.54±0.51	3.46±0.06a**b**
Group IV	4.51±1.7	4.52±0.7	1.9±0.41	2.20±0.35 c**
Group V	2.64± 1.5	2.03±0.54	1.66±1.05	0.56±0.17 c**

Each value is the mean ±SEM for 4 animals, a-indicates significant difference with normal control groups, b- indicates significant difference with cystone treated groups, c-indicates significant difference with lithiatic control groups. *- P<0.05, **-P<0.01.

Table 3. Effect of MFNC on serum biochemical parameters.

Treatment groups	Urea mg/dl	BUN mg/dl	Uric acid mg/dl	Creatinine mg/dl
Group 1	32.87±1.15	15.20±0.54	2.58±1.08	1.49±0.16
Group II	62.32±13.21a*	29.11±6.17a*	1.06±0.32	1.57±0.06
Group III	109.01±6.54a**	50.94±3.05a**b**	14.36±0.26a**	2.11±0.26
Group IV	43.73±1.93b*c**	20.17±0.88b*c**	4.10±0.94c**	0.55±0.03a**b**c**
Group V	38.65±0.49b*c**	19.93±1.16b*c**	4.90±0.36c**	1.98±0.20

Each value is the mean ±SEM for 4 animals, a-indicates significant difference with normal control groups, b- indicates significant difference with cystone treated groups, c-indicates significant difference with lithiatic control groups. *- P<0.05, **-P<0.01.

Table 4. Effect of MFNC on kidney.

Treatment groups	Protein (mg/24hr)	Phosphorus (mg/24hr)	Calcium (mg/24hr)	Oxalate (mg/24hr)
Group1	5.003±0.393	2.54±0.424	0.62±0.63	0.15±0.01
Group II	5.373±0.037	3.82±0.109c**	1.06±0.21c**	2.92±0.32a**
Group III	4.733±0.296	14.77±1.297a*	12.47±0.83	3.53±0.10a**
Group IV	4.800±0.404	3.69±0.081b**	0.27±0.24a**c**	0.37±0.14b**c**
Group V	4.900±0.252	4.82±0.313 b**	1.84±0.38a**c**	0.37±0.05b**c**

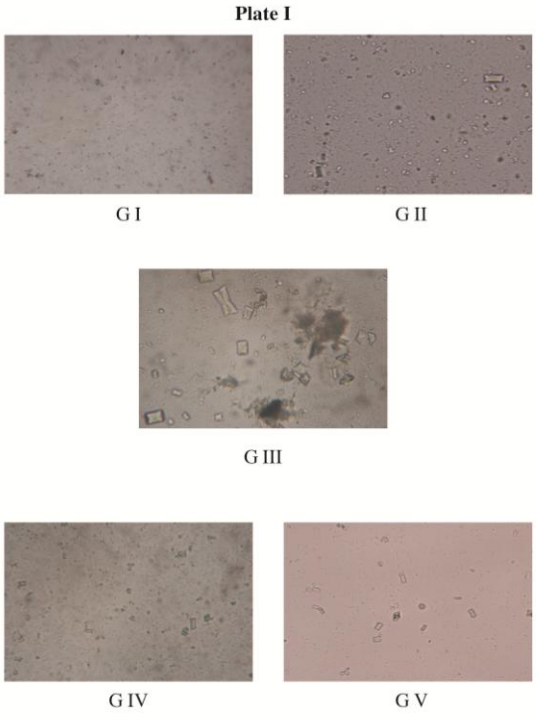
Each value is the mean ±SEM for 4 animals, a-indicates significant difference with normal control groups, b- indicates significant difference with cystone treated groups, c-indicates significant difference with lithiatic control groups. *- P<0.05, **-P<0.01.

The urine microcrystal examination of all the fruit extract treated groups of rats exhibited decreased crystalluria than ethylene glycol treated group. Even after the treatment with MFNC the type of the stone and amount of stones in urine were reduced (Plate I). When compared to group III, group V showed reduced amount of crystalluria. Few struvite stones, calcium oxalate crystals and small fragmented stones were present in the urine samples of EG/AC administrated group. The calcium oxalate monohydrate struvite and phosphate stones which are observed in group III were absent in urine samples from group IV and V.

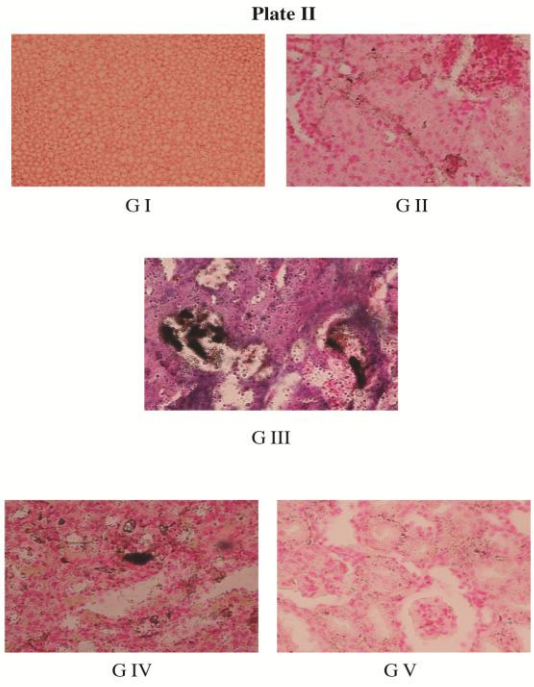
Special staining of the kidney tissues of all MFNC administered rats strengthens the observations from the urine and serum analysis. The amount of deposition of calcium oxalate stones was very less in group IV and V when compared to group III in which large stones were dispersed all regions and the stones were concentrically arranged around the renal tubules and are seen as black dots and some stones were seen inside the tubules also. Group V, received the highest dose of MFNC was noticed with less depositions of stones in the lumen as well as in the luminal epithelium of kidney when compared to all other treated groups of rats (Plate II). Among the MFNC treated groups, high dose administered group (group V) was found to have more curative effect than group IV.

Discussion

Here, fruit extracts of *N. cadamba* has been explored to evaluate its urinary protection in standard nephrolithiatic model. The stone inducing agents have increased the urinary oxalate and calcium in group III (lithiatic control) treated with EG/AC. The observed results are in agreement with previous studies which indicated that EG administration develops renal calculi which composed mainly of calcium oxalate [6]. The increased urinary calcium enhance the process of nucleation and precipitation of calcium and oxalate which causes calcium oxalate or calcium phosphate stones [7]. The decreased calcium and phosphorus levels in methanol fruit extract of *N. cadamba* (MFNC) is evident for the action of the extract in curing calcium phosphate stones.



Urine micro crystals of treatment groups (28th day)



Silver nitrate staining of treatment groups

Findings of previous studies have showed that the elevated oxalate and phosphate excretion induced the formation of calcium phosphate and calcium oxalate stones [8]. The results confirm the curative property of methanol fruit extract in calcium oxalate and calcium phosphate excretion.

Several studies demonstrated that inter α inhibitor (IαI) family proteins and bikunin are the major proteins in the stone matrix and presence of these proteins modulate the process of crystal aggregation. The lowered urinary protein concentration by the methanol extract may reduce the formation of stone matrix or crystal aggregation. The data revealed that fruit extract could regulate the proteins involved in hyperoxaluria and crystal deposition. The methanol extract of *N. cadamba* provide an inhibitory role in regulating the process of crystallization by reducing crystal forming substances.

The increased nitrogenous waste materials in calculi induced rats (EG/AC administered groups) are due to the reduced glomerular filtration rate (GFR) which obstruct the urine flow by the deposition and block renal tubules with stones [9]. The present study also demonstrated that methanolic extract reduces the serum nitrogenous waste material either by increasing glomerular filtration rate or by reducing the deposition of calcium oxalate stones.

Silver nitrate staining staining exhibited positive results and stones were located in the lumen as well as in the epithelial lining of the tubules. Crystal depositions severely affected some areas and cause inflammation which leads to the loss of structural integrity and glomerular atrophied and the urinary space is occupied by crystals. The administration of MFNC to urolithiatic rats reduced the deposition of crystals in urine and kidney and the extract is found to have curative effect against kidney stone. Urine microcrystal analysis revealed that MFNC can reduce crystalluria. Calcium oxalate crystals are found to be reduced by MFNC administration as evident from Pizzalto's staining.

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