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Research Article

EVALUATION OF ANTIUROLITHIATIC ACTIVITY OF ETHANOLIC EXTRACT OF *ANNONA RETICULATA* AGAINST ETHYLENE GLYCOL AND AMMONIUM CHLORIDE INDUCED UROLITHIASIS IN ALBINO WISTAR RATS

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ABSTRACT
Urinary stone disorder has afflicted humankind since antiquity and can persist, with serious medical consequences, throughout a patient's lifetime. The common component of urinary stone is calcium oxalate (CaOx). In spite of tremendous advances in the field of medicine, there is no truly satisfactory drug for the treatment of renal calculi. In the indigenous system of medicine, the leaves of *Annona reticulata* (Family - Anonaceae) are reported to be useful in the treatment of urinary stones. Hence, in the present study, the Annona reticulata have been selected for their anti urolithiatic activity on experimentally induced urolithiatic rats.
Keywords: Ammonium chloride, Ethylene glycol, Lithiasis, *Annona reticulata.*

INTRODUCTION

Traditional medicine using herbal drugs exists in every part of the world. The major areas are Chinese, Indian and European traditions[1]. The philosophies of these traditional medicines have some resemblance to each other but differ widely from modern Western medicine. The development procedure of herbal drugs for world-wide use has to be different from that of synthetic drugs[2]. Thus, the Indian Medical System-Ayurveda came into existence. The raw materials for Ayurvedic medicines were mostly obtained from plant sources in the form of crude drugs such as dried herbal powders or their extracts or mixture of products[3]. Also, Siddha, Unani and Tibb are traditional health care systems have been flourishing for many centuries. Apart from these systems there has been a rich heritage of ethno botanical usage of herbs by various colourful tribal communities in the country[4].

MATERIAL AND METHOD

The *Annona reticulata* were shade dried at room temperature and coarsely powdered in such a way that the material passed through sieve no. 20 and was retained on sieve no. 40 for desired particle size and then extracted with 80 %v/v alcohol, separately.

Collection and authentication

The plant was collected during the march 2014 from Sri Venkateshwara University Tirupati, India. The plant was authenticated by Dr. Madhava Chetty, Department of Botany and voucher specimen of the plant were preserved at institute herbarium library.

Determination of foreign matter

Foreign matter in herbal drugs consists of either parts of the medicinal plant or it may be any organism, part or product of an organism. It may also include mineral admixtures not adhering to the medicinal plant materials e.g. soil, stones, dust etc. The specified quantity of plant material is spread on a thin layer of paper. By visual inspection or by using a magnifying lens (5X or 10X), the foreign matters are picked out and the percentage is recorded.

Animal selection

Wistar albino rats of either sex weighing between 150 and 200 g were selected for acute toxicity studies and for the anti urolithiatic activity. The animals were acclimatized to standard laboratory conditions of temperature (22 ± 3^0C) and maintained on 12:12 h light: dark cycle. They were provided with regular rat chow and distilled water *ad libitum.* The animal care and experimental protocols were in accordance with CPCSEA / IAEC. Research proposal approval no. is GSP/IAEC/2014/02/02.

Chemicals used

Ethylene glycol (AR Grade) was obtained from Merck Laboratories, Mumbai, India, Cystone were used as standard anti urolithiatic drug.

Extracts used

Ethanolic extract of *Annona reticulata* were of were suspended in distilled water (q.s.) using 2 % tween 80 as a suspending agent. The extracts were subjected to acute oral toxicity study and depending upon LD_{50}, the calculated quantity of each extract was given to each animal in corresponding group, once daily, through per oral route.

Preparation of alcoholic extract

About 200 g of dried powder was extracted with 80 % v/v alcohol in a soxhlet extractor. The extraction was continued until the solvent in the thimble became clear. After

complete extraction, the extract was filtered and solvent was distilled off. The extract was concentrated to dry residue. The percentage yield of the extract was calculated with reference to air dried powder.

Acute toxicity study

The acute oral toxicity study was carried out as per the guidelines set by organization for economic co-operation and development (OECD) revised draft guidelines 423 B ("Up and Down" method) received from committee for the purpose of control and supervision of experiments on animals (CPCSEA), Ministry of Social Justice and Empowerment, Government of India. The LD_{50} cut-off dose for EE are $1/5^{th}$ and$1/10^{th}$ of the LD_{50} dose was taken as a therapeutic dose. Acute toxicity study was carried out using "Up and Down" method. Male Wistar albino rats were used for assessment of anti urolithiatic activity. Ethylene glycol and ammonium chloride induced hyperoxaluria model was used to induce urolithiasis. Thirty animals were randomly divided into five groups containing six animals in each. Group I served as a vehicle treated control and maintained on regular rat food and drinking water *ad libitum*.

Screening Method / Model used

Ethylene glycol induced urolithiasis model in albino rats

Animals were divided in 5 groups containing six in each and kept in metabolic cages. All animals had free access to regular rat chow and drinking water *ad libitum* for 28 days. Renal calculi were induced in group II to V by supplementing with 0.75 %v/v ethylene Glycol and ammonium chloride in drinking water *ad libitum*. Group IV to V were treated with plant extracts starting from 1^{st} day to 28^{th} day (Preventive regimen). Ethylene glycol (0.75 %) in drinking water was fed to groups II-V for induction of renal calculi until the 34^{th} day. As well as ethylene glycol, groups 2-6 also received the following treatments:

Groups III received standard anti urolithiatic drug, cystone (750 mg/kg body weight).
Group IV received ethanolic extract of *Annona reticulata* (200 mg/kg body weight)
Group V received ethanolic extract of *Annona reticulata* (400 mg/kg body weight).

All extracts were given once daily by oral route. After 28 days, urine 24 h was analyzed for oxalate, calcium and phosphate excretion. Kidneys were isolated .Serum levels of creatinine and uric acid was also estimated. Histopathology of kidney was also studied[5-7].

Table 1: The treatment with *Annona reticulata* extract significantly ($P < 0.001$) elevated the serum levels of creatinine, calcium, phosphate, urea and uric acid

Treated Groups	Serum Parameters (mg%)				
	Urea	Uric Acid	Creatinine	Calcium	Phosphorus
Normal (0.9 % saline)	41.65 ± 0.528	2.63 ± 0.130	0.426 ± 0.018	12.746 ± 0.257	3.668 ± 0.266
Control (EG 0.75 % + 2 % AC)	72.10 ± 0.538[a]	7.21 ± 0.247[a]	0.918 ± 0.025[b]	30.848 ± 0.64[b]	11.006 ± 0.435[c]
Standard (cystone 5 mg/kg)	41.27 ± 0.500[***]	2.91 ± 0.146[***]	0.536 ± 0.027[*]	12.381 ± 0.426[**]	5.933 ± 0.174[**]
EEAR1 (200 mg/kg)	53.25 ± 0.572[***]	4.12 ± 0.251[***]	0.781 ± 0.028[**]	15.151 ± 0.231[*]	8.523 ± 0.318[*]
EEAR2 (400 mg/kg)	45.93 ± 0.652[***]	3.35 ± 0.302[*]	0.551 ± 0.021[*]	13.29 ± 0.369[*]	7.038 ± 0.256[*]

Table 2: The treatment with *Annona reticulata* extract significantly ($P < 0.001$) elevated the urine levels of creatinine, calcium, phosphate, urea and uric acid (Units of all the parameters are mg%)

Treated Groups	Urine Parameters (mg%)				
	Urea	Uric Acid	Creatinine	Calcium	Phosphorus
Normal (0.9 % saline)	12.49 ± 0.263	10.27 ± 0.363	14.58 ± 0.390	32.04 ± 1.233	3.68 ± 0.199
Control (EG 0.75 % + 2 %AC)	24.13 ± 0.41[a]	27.82 ± 0.37[a]	29.51 ± 0.468[a]	34.15 ± 1.36[a]	9.39 ± 0.218[a]
Standard (cystone 5 mg/kg)	14.78 ± 0.333[***]	11 ± 0.335[***]	16.37 ± 0.365[***]	34.28 ± 1.030[***]	4.77 ± 0.227[**]
EEAR1 (200 mg/kg)	16.22 ± 0.270[***]	16.42 ± 0.330[***]	21.51 ± 0.580[**]	36.99 ± 0.754[***]	7.56 ± 0.283[**]
EEAR2 (400 mg/kg)	14.70 ± 0.379[***]	12.56 ± 0.244[***]	17.21 ± 0.553[***]	34.29 ± 1.082[***]	5.74 ± 0.217[*]

Table 3: The treatment with *Annona reticulata* extract significantly ($P < 0.001$) showed an increase in urine volume, urine pH and kidney weight (Units of all the parameters are mg%)

Treated Groups	Other Parameters		
	Urine Volume (mg%)	Urine P^H	Kidney Weight (mg%)
Normal (0.9 % saline)	17.10 ± 1.08	7.0 ± 0.20	0.60 ± 0.08
Control (EG 0.75 % + 2 %AC)	7.57 ± 0.5[a]	5.23 ± 0.39[b]	0.52 ± 0.09[c]
Standard (cystone 5 mg/kg)	12.95 ± 0.88[***]	7.79 ± 0.35[**]	0.62 ± 0.09[*]
EEAR -1 (200 mg/kg)	8.85 ± 0.78[ns]	6.36 ± 0.39[ns]	0.60 ± 0.11[***]
EEAR-2 (400 mg/kg)	10.84 ± 0.76[*]	7.67 ± 0.15[***]	0.73 ± 0.12[***]

RESULT AND DISCUSSION

The dose of 2000 mg/kg b.w. for the extracts of *Annona reticulata* was found to be safe. Hence, therapeutic dose was taken as 200 mg/kg b.w and 400 mg/kg b.w. The treatment with *Annona reticulata* extract significantly ($P < 0.001$) elevated the serum levels of Creatinine, calcium, phosphate, urea and uric acid. The histopathological study of the kidney also indicated the above results. The results were comparable to that of standard drug cystone. In the present study, dried powder of *Annona reticulata* extracted with 80 %v/v alcohol in this study, male rats were selected to induce urolithiasis because the urinary system of male rats resembles that of humans[8] and earlier studies have shown that the amount of stone deposition in female rats was significantly less[9]. Acute toxicity study by "Up and Down" method showed the LD_{50} cut-off doses at 2000 mg/kg body weight, indicating that the drug is much safer. $1/5^{th}$ and $1/10^{th}$ of LD_{50} dose was used as a therapeutic dose for anti urolithiatic and antiulcer activity. Urinary stone formation takes place due to changes in urinary chemistry, such as ashyperoxaluria and hypercalciuria, leading to urinary super saturation, which later crystallizes, aggregates and ends up in stone formation[10-12]. Evidences in previous studies indicated that, in response to 14 days period of ethylene glycol (0.75 %v/v) and ammonium chloride administration in young albino rats forms renal calculi composed mainly of CaOx. The principal precursor of oxalic acid in mammals is glyoxalic acid[13]. The enzymatic oxidative conversion of glycolate to oxalate via glyoxylate is the major metabolic pathway involved in endogenous oxalate synthesis. The enzymatic disturbances are the causative factors for the idiopathic hyperoxaluria; while, the defective intestinal absorption of oxalate plays a vital role in enteric hyperoxaluria and lead to an increase in the urinary oxalate concentration[14] In the present study, chronic administration of 0.75 % ethylene glycol aqueous solution to male Wistar rats resulted in hyperoxaluria. Oxalate and calcium excretion in urine were grossly increased in calculi-induced animals. Since it has been already proved that hyperoxaluria is posing significant risk in the pathogenesis of renal stones as compared with hypercalciuria, the changes in urinary oxalate levels are relatively more important than those of calcium[15]. Increased urinary calcium is facilitating the nucleation and precipitation of CaOx or apatite (CaPh) in urine and subsequent crystal growth[16]. The increase in calcium retention of kidney and its urinary excretion may be due to defective renal tubular reabsorption[17] of the same or an increase in absorption from the intestine as the patients with renal calcium stones are reported to have increased absorption of calcium[18]. *Annona reticulata* lowers the levels of oxalate and calcium in urine and their retention in kidney also reduced significantly.

Remarkable increase in urinary phosphate was also observed in calculi-induced rats. Increased urinary phosphate excretion along with oxalate stress seems to provide an environment appropriate for stone formation by forming CaPh crystals, which epitaxially induces CaOx deposition[19]. Treatment with *Annona reticulata* extract restores urinary phosphate level, thereby reducing the risk of stone formation. In urolithiasis, the glomerular filtration rate (GFR) decreases due to the obstruction to the outflow of urine by stones in urinary system. Due to which, the waste products, particularly nitrogenous substances such as urea, creatinine and uric acid gets accumulated in blood[20]. Additionally, increased lipid peroxidation and decreased levels of antioxidant potential have been reported in the kidneys of rats supplemented with a calculi-producing diet[21,22]. In this context, oxalate has been reported to induce lipid peroxidation and to cause renal tissue damage by reacting with polyunsaturated fatty acids in cell membrane[23]. In calculi-induced rats, the elevated serum levels of creatinine and uric acid, phosphate and calcium indicate marked renal damage. *Annona reticulata* hastens the process of dissolving the preformed stones in prevention of new stone formation in urinary system on prophylactic treatment. The histopathological study[24] supported the results. The markedly elevated serum levels of creatinine and uric acid in stone-forming animals were indicative of prominent necrosis of renal epithelia. In the calculi induced animals, there was damage to the end part of nephron and collecting system. Elevated levels of oxalate in urine and even its retention in kidney may be one of the causative factors for the peroxidative degeneration of renal epithelia[25]. However, preventive treatment with *Annona reticulata* prevents oxalate induced lipid peroxidation and causes regeneration of renal epithelium.

CONCLUSION

The presented data indicate that administration of *Annona reticulata* extract to rats with experimentally-induced urolithiasis reduced and also prevented the formation of urinary stones. The reduction in the stone forming constituents in urine brought about by *Annona reticulata* could contribute to its anti urolithiatic property.

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