IJPT
1735-2657/14/131-35-39
IRANIAN JOURNAL OF PHARMACOLOGY & THERAPEUTICS

IJPT 13: 35-39, 2014

ORIGINAL ARTICLE

Effect of *Citrus Medica* Linn. in Urolithiasis Induced by Ethylene Glycol Model

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Received May 16, 2013; Revised July 5, 2013; Accepted August 14, 2013

This paper is available online at *http://ijpt.tums.ac.ir*

ABSTRACT

The Objective of this study is to study the Effect of Citrus medica in urolithiasis induced by Ethylene Glycol model. A number of 24 Male Wistar albino rats (250-300 g) were divided in to 4 groups namely Group I (Normal control), Group II (Model control), Group III (Standard-treated, cystone – 750 mg/kg) and Group IV (Citrus medica – 250 mg/kg). Except group I, Ethylene glycol (0.75% v/v in drinking water) was provided for each group of animals for 28 days study period. After the completion of treatment, various physical parameters (body weight, diuresis, pH), level of urolithiatic promoters (calcium, oxalate, Inorganic phosphate and uric acid) and urolithiatic inhibitors (magnesium and citrate) were measured in 24-h urine, blood samples and kidney homogenate. Antioxidant parameters & renal function tests were carried out as well. Both cystone and extract of Citrus medica showed significant increase in physical parameters and stone forming inhibitors and significant decrease in stone forming promoters. Degree of oxidative stress reduced with cystone and in treatment group. Extract of Citrus medica Linn. showed significant activity against urolithiasis which is due to diuretic and antioxidant activity and its ability to increase inhibitors and decrease promoters levels.

Keywords: *Urolithiasis, Citrus medica, Ethylene glyco*

Herbs are staging a comeback and herbal 'renaissance' is happening all over the globe. The herbal products today symbolize safety in contrast to the synthetics that are regarded as unsafe to human and environment. Although herbs had been priced for their medicinal, flavouring and aromatic qualities for centuries, the synthetic products of the modern age surpassed their importance, for a while. Traditional systems of medicine continue to be widely practiced on many accounts. Population rise, inadequate supply of drugs, prohibitive cost of treatments, side effects of several allopathic drugs and development of resistance to currently-used drugs for infectious diseases have led to increased emphasis on the use of plant materials as a source of medicines for a wide variety of human ailments. However, the blind dependence on synthetics is over and people are returning to the naturals with hope of safety and security.

Urolithiasis has been a perplexing problem since the dawn of history. The process of stone formation in any part of urinary tract including kidney, urethra, ureters and urinary bladder is called Urolithiasis. In India, 12% of the population is expected to have urinary stones, out of which 50% may end up with loss of kidneys or renal damage [1]. The aetiology of urinary calculus disease is quite complex and variable. Involvement of genetic, metabolic, dietary factors, anatomical defects [2] and temporal, geographical and individual variations [3] further complicate it.

Therapy with decreased calcium and oxalate intake, thiazides (Diuretics), phosphate salts and allopurinol in various combinations has substantially decreased the prevalence of recurrent stones. But these drugs have some adverse effects which limit their use in long-term medical treatment [4]. Inspite of intensive research to establish the mechanisms of stone formation, dietary

management, evaluation of medicinal plants and other agents in the treatment of urinary stones, still to date there is no standard drug available. Numerous drugs, diuretics and antispasmodics are used in order to facilitate the passage and expulsion of calculi but management of urinary calculi is still far from satisfactory. With the limitations of medical treatment, surgical removal of stone is still considered the most successful way to relieve the symptoms, but having disadvantage of high cost and recurrence rate. So keeping in view the above facts, search for an ideal antiurolithiatic drug continues and a trial should be given to indigenous drugs.

Green plants synthesize and preserve a variety of biochemical products, many of which are extractable and used as chemical feed stocks or as raw material for various scientific investigations. In the present scenario, the need for basic scientific investigations on medicinal plants used in the indigenous systems becomes imminent. *Citrus medica* Linn. (Rutaceae) commonly known as bijaura fruit, has very good antioxidant activity [5]. It has beneficial effect on renal calculi. Besides that, it has good activity in various conditions like constipation, improper secretion of bile juices, nausea, emesis and piles. With this background, the objective of the present study is to evaluate antiurolithiatic activity of *Citrus medica* Linn. in rats implanted with calcium oxalate seed.

MATERIALS AND METHODS

Collection of plant material

Fresh fruits of *Citrus medica* Linn. were collected from botanical garden of National Research Centre, Boriavi-Anand, Gujarat, India. The plant was identified and authenticated by Dr. Hitesh A. Solanki, Reader, Department of Botany, Gujarat, Ahmedabad. A Voucher specimen APCH 11 has been deposited at the herbarium.

Preparation of test drug

Fresh juice was collected by squeezing the fruit before each dosing and filtered with muslin cloth. (0.4#)

Animals

Healthy adult male Wistar Albino rats (250-300gm) were selected for study of antiurolithiatic activity. The animals were acclimatized to standard laboratory conditions (temperature: 25 ± 5°C), humidity (55 ± 5%) and maintained on 12-h light: 12-h dark cycle. They were provided with regular rat chow and drinking water *ad libitum.*

The experimental protocol was approved by Institutional Animal Ethical Committee as per the guidance of Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Ministry of Social Justice and Empowerment, Government of India (Protocol No. 7014)

Ethylene glycol induced urolithiasis (Preventive study) [6]

Thirty rats were randomly divided in five groups – Group I (normal control), Group II (Model control), Group III (standard-treated, cystone – 750 mg/kg) and Group IV (test treated, citrus medica – 250 mg/kg) containing six rats each. Urolithiasis was induced in animals of all groups except group I by administration of ethylene glycol in concentration of 0.75 % v/v in drinking water for 28 days. Additionally, animals of group III and IV received cystone (750 mg/kg, p.o) and prophylactic dose of *Citrus medica (250 mg/kg, p.o)* throughout the study period.

Collection and analysis of urine samples

All animals were kept in individual metabolic cages and 24-h urine samples were collected on 28th day. Animals had free access to drinking water during the urine collection period. After collection of urine, pH of urine was measured [7] and then drop of concentrated hydrochloric acid was added to the urine before being stored at 4^{o}C. Different elements like calcium, oxalate, magnesium were measured [8-9]. Urine was also analyzed for various biochemical parameters like uric acid, citrate, inorganic phosphate [10]. To determine oxidative stress, lipid peroxidation level and catalase were also measured.

After the experimental period, blood was collected by heart puncture under anesthetic conditions and animals were sacrificed by cervical decapitation. Serum was separated by centrifugation at 15000 rpm for 20 min and analyzed for different biochemical parameters like BUN and creatinine and elements like calcium [11].

Assay of Renal Tissue Samples

The abdomen was cut open to remove both kidneys from each animal. Isolated kidneys were cleaned off extraneous tissue. One kidney from each animal was weighed and used to prepare homogenate in phosphate buffer (pH 7). Another kidney from each animal was dried at 80^{o}C in a hot air oven and weighed. A sample of 100 mg of the dried kidney was boiled in 10 mL of 1N hydrochloric acid for 30 min and homogenized. The homogenate was centrifuged at 10000 rpm for 20 min and the supernatant was separated [12]. Kidney homogenate was used for estimation of catalase and MDA [13-14].

Statistical analysis

The values are expressed as mean ± SEM. The statistical analysis was carried out by one way analysis of variance (ANOVA). The $p \leq 0.05$ was considered significant.

Published online: January 28, 2014

Table 1. General variables measured after 28 days of treatment

Variables	Normal control	Model Control	Standard (Cystone) (750 mg/kg)	*Citrus medica* (250 mg/kg)
pH	6.61 ± 0.05	9.25# ± 0.05	7.65* ± 0.04	7.98* ± 0.10
Diuresis (ml)	18.17 ± 0.872	16.83 # ± 1.4	20.67* ± 1.085	22.17* ± 0.872
% Change in weight	10.14 ± 0.28	-3.33 # ± 0.08	2.61* ± 0.10	2.50* ± 0.02
Wet kidney weight (mg)	0.77 ± 0.008	1.14# ± 0.053	0.83* ± 0.003	0.88* ± 0.010
Dry kidney weight (mg)	0.19 ± 0.003	0.30# ± 0.007	0.21* ± 0.005	0.20* ± 0.005
Urine Analysis				
(a) Urinary Excretion of Lithogenic Promoters				
Calcium (mg/24hr)	1.39 ± 0.02	4.80# ± 0.18	2.13* ± 0.02	2.06* ± 0.32
Oxalate (mg/24hr)	3.03 ± 0.05	11.60#± 0.86	6.70* ± 0.15	6.52* ± 0.14
Inorganic Phosphate (IP) (mg/24hr)	6.44 ± 0.10	9.56# ± 0.70	7.14* ± 0.06	8.11* ± 0.32
Uric acid (mg/24hr)	1.82 ± 0.08	4.70# ± 0.18	2.92* ± 0.09	2.76* ± 0.16
(b) Urinary Excretion of Lithogenic Inhibitors				
Citrate (mEq/L)	16.91 ± 0.24	15.94# ± 0.11	29.90* ± 0.23	24.21** ± 0.45
Magnesium (mEq/L)	1.20 ± 0.06	1.13 ± 0.08	3.90* ± 0.19	4.49**± 0.45
Serum Analysis				
BUN (mg/dl)	31.24 ± 0.91	57.12 # ± 0.35	37.00* ± 0.13	39.13* ± 0.16
Creatinine Clearance (ml/min)	0.21 ± 0.02	0.14# ± 0.01	0.36* ± 0.015	0.33* ± 0.04
Calcium (mg/dl)	9.51 ± 0.21	13.20# ± 0.13	9.50* ± 0.03	8.48* ± 0.27
Kidney Homogenate analysis				
Catalase (mg/100 mg of kidney)	82.60 ± 6.20	5.15# ± 5.77	86.97*± 4.25	86.20 *± 3.93
MDA (µg/ml)	0.81 ± 0.00	1.31# ± 0.01	0.88* ± 0.00	0.89* ± 0.03

All values are expressed as Mean ± SEM (N = 6)
Statistical analysis was carried out using one-way ANOVA test.
#Significant difference from normal control $p \leq 0.05$
*Significant difference from model control $p \leq 0.05$

RESULTS

There was significant decrease in animal weight and urine volume per day and increase in the dry and wet kidney weight in model control as compared to normal control animals. These changes were significantly prevented by treatment with standard (750 mg/kg; p.o.) and Test drug (250 mg/kg; p.o.). Urinary pH was found acidic in normal control whereas alkaline in model control. Treatment with standard and test drug significantly prevented this shift of pH from acidic to alkaline (Table 1).

All urolithiatic promoters' level in each biological sample was significantly increased in model control as compared to normal control. Treatment of standard and test drug significantly prevented these rise in promoters level (Table 1).

Ethylene glycol consumption for 28 days produced no significant change in level of magnesium in urine and serum sample, in model control as compared to normal control. But citrate level was significantly reduced in urine of model control as compared to normal control. Treatment with standard and test drug prevented reduction in the level of inhibitors as compared to model control. It was also observed that treatment with standard and the test drug lead to increase in inhibitors level as compared to normal control group (Table 1).

Crystal formation by ethylene glycol leads to impairment of renal function which results in marked increase in BUN and decrease in creatinine clearance in urine. Similar results were also observed in our study in model control group. Treatment with standard and test drugs significantly improved renal function reveled from BUN and creatinine clearance data. (Table 1)

Exposure of ethylene glycol for 28 days significantly decreased the catalase level and increased MDA level in model control group as compared to normal control. While treatment with standard and test drug showed significant rise in catalase level and decrease in MDA level. (Table 1)

DISCUSSION

Stones with smaller size can easily travel through the urinary system, but bigger size ones may lead to obstruction and pain. This could also result in decrease in food consumption [15]. The present study also revealed a similar pattern with significant reduction in body weight in model control group. Standard and test

drug treatment might have prevented stone aggregation and thus relieved animals from pain. Urine is usually acidic which prevents stone formation whereas alkalinization of urine favors stone aggregation. Shift towards alkaline pH was observed in model control animals providing suitable environment for stone formation [16]. Treatment with Standard and test drug prevented this shift in pH towards alkaline.

Increased urinary calcium concentration is a factor favoring the nucleation and precipitation of calcium oxalate or apatite (calcium phosphate) from urine and subsequent crystal growth. Crystallization and aggregation of lithogenic substances like ethylene glycol leads to calculi formation. It is proved by researchers that increased calcium and oxalate levels promote stone formation [17]. In the study, oxalate and calcium excretion were progressively increased in model control group animals, but treatment with cystone and *Citrus medica* decreased oxalate and calcium levels. This reduction in excretion of oxalate and calcium is beneficial in preventing stone formation or growth by reducing supersaturation of the urine.

Increased excretion of inorganic phosphate and uric acid has been reported in stone formers [18] and hyperoxaluric rats [19]. 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 [20]. Uric acid interferes with calcium oxalate solubility [21] and it binds and reduces the inhibitory activity of glycosaminoglycans [21]. There was significant increase in excretion of inorganic phosphate & uric acid in serum and urine in the model control compared with normal control group but this was significantly prevented with the treatment with standard and test drug.

Magnesium is considered as urolithiatic inhibitor [22]. It decreases the growth and nucleation rate of calcium oxalate crystals by interfering with absorption of oxalate [23]. In model control animals, magnesium concentration in urine samples was not altered but standard and test drug administration significantly increased magnesium levels in biological samples as compared to model control. Citrate is an important urolithiasis inhibitor, which forms soluble complexes with calcium and inhibits precipitation and aggregation of calcium oxalate and phosphate [24]. Standard and test drug treatment leaded to increase in citrate concentration, which may be responsible for protective effect.

Presence of crystal aggregation in the kidney causes obstruction in the kidney‘s normal function and resulting in decreased creatinine clearance. Another reason for this damage to glomerulus and tubules is systemic generation of reactive oxygen species. Selvam [25] in 2002 also reported calcium oxalates crystals cause oxidative stress. Huang *et al.* [26] and Pippenger *et al.* [27] have suggested that oxidative stress can be compensated by elevated antioxidant enzymes like catalase in the kidney. Administration of standard and test drug resulted in significant decrease in lipid peroxidation and significantly prevented decrease in the catalase levels in kidney as compared to the model control animals, which suggests its efficacy in preventing free radical-induced damage.

In conclusion, C*itrus medica* Linn. was effective in prevention of crystal aggregation which may be result of combination of various effects like decrease in promoters level, increase in inhibitors level and its diuretic and antioxidant activity.

Published online: January 28, 2014

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