A polyherbal formulation attenuates hyperoxaluria-induced oxidative stress and prevents subsequent deposition of calcium oxalate crystals and renal cell injury in rat kidneys

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[ABSTRACT] INTRODUCTION: Cystone is an approved Ayurvedic polyherbal proprietary medicine used in India for various urinary disorders, including urolithiasis. AIM: To evaluate the protective effect of Cystone against hyperoxaluria-induced oxidative stress and calcium oxalate crystal deposition in urolithiasis. METHODS: Ethylene glycol (EG) (0.75%, V/V) in drinking water was given to rats for 28 days to induce urolithiasis with simultaneous treatment of Cystone (500 and 750 mg/kg body weight), and various urinary risk factors of urolithiasis and antioxidant markers were assessed. RESULTS: EG treatment lead to increased urine volume and lowered urinary pH, along with increased urinary excretion of oxalate, calcium and phosphate in untreated animals. These changes caused extensive calcium oxalate crystal deposition, increased lipid peroxidation and decreased activity of antioxidant enzymes (SOD, catalase and GPx) in the kidney of untreated rats. Cystone prevented these hyperoxaluric manifestations and inhibited calcium oxalate crystal deposition in treated rats at both doses. CONCLUSIONS: Cystone therapy provides protection against hyperoxaluria-induced oxidative stress and calcium oxalate crystal deposition by improving renal tissue antioxidant status and diuresis.

[KEY WORDS] Urolithiasis; Hyperoxaluria; Oxidative stress; Ethylene glycol; Calcium oxalate; Ayurveda, polyherbal

1 Introduction

Cystone is an Ayurvedic, polyherbal, proprietary medicine approved by the drug regulatory authority, Department of AYUSH, Ministry of Health and Family Welfare, Government of India. It has been clinically used extensively for treating many urinary tract complications, such as urolithiasis, burning micturition, urinary tract complications in pregnancy and other various renal disorders [1]. The Indian Ayurvedic system of medicine mentioned Pashanabheda (Pashana-stone; Bheda-break) for the treatment of urolithiasis. Pashanabheda is a group of plants with diuretic and anti-urolithiatic activities (e.g. *Bergenia ligulata* (Wall.) Engl.), and Cystone contains some of these as ingredients, as indicated in the Materials and Methods section.

Urolithiasis is a recurrent renal disease which affects 4%–8% of the population in the UK, 15% in the US, 20% in the Gulf countries and 11% of the population in India [2-3]. Epidemiological data suggest that 60%–80% of renal stones is composed of calcium oxalate (CaOx). Stone formation tends to recur; without preventative measures after a first stone, at the rate of 40% within 3 years, and up to 75% by 10 years. Within 25 years virtually every patient has formed at least one more stone [3]. In Ayurveda, urolithiasis is compared to "Mutrashmari", which is a stone-like structure anywhere in the mutravaha srotas (urinary system). Which means mutra=urine, ashma=stone, ari=enemy. The formation of a renal stone, which causes great pain and suffering to the body, like an enemy, is called "Mutrashmari". Ayurveda suggests that this disorder is due to vitiation of all doshas affecting the urinary system. Oxalate stones are formed predominantly due to high vata vitiation. Pathophysiology suggests that hyperoxaluria is one of the major risk factors for human idiopathic CaOx urolithiasis. Oxalate is a natural by-product of metabolism and is excreted harmlessly in normal individuals. However, increased urinary excretion of oxalate can be toxic largely because of its propensity to crystallize at physiologic pH and form CaOx [4]. Exposure to oxalate/CaOx generates toxic responses in renal epithelial cells, including altered membrane surface properties and cellular lipids, changes in
gene expression, disruption of mitochondrial function, formation of reactive oxygen species (ROS) and decreased cell viability [5]. An overproduction of ROS and a reduction in cellular antioxidant capacities leads to the development of oxidative stress [3]. While enzymatic and non-enzymatic systems preserve the antioxidant status, these defense systems become overwhelmed during oxidative stress and this leads to diminished antioxidant capacity in renal tissue [6]. Loss of membrane integrity subsequently facilitates the retention of CaOx crystals and the growth of stones in renal tubules [7]. Drugs with multiple mechanisms of protective action, including antioxidant properties, may be one way forward in minimizing this type of tissue injury in urolithiasis [8]. It has been demonstrated that many plant-based formulations possess potent anti-oxidant actions, and are effective in healing experimentally-induced urolithiasis. From these findings, Cystone is expected to be effective in preventing stone formation and in healing hyperoxaluric oxidative stress-induced renal cell injury. Thus, the present study was designed to evaluate the mechanism of the protective effect of Cystone in hyperoxaluria-induced calcium oxalate urolithiasis.

2 Material and Methods

2.1 Composition of Cystone

Cystone syrup (The Himalaya Drug Company, Bangalore, India) was purchased from the local drug store, Ranchi. Cystone liquid concentrate (5 mL) contains extracts of the following medicinal plants in definite proportions: Gokshura (Tribulus terrestris L.) 91 mg; Punarnava (Boerhavia diffusa L.) 67 mg; Pashanabheda (Saxifraga ligulata Murray) 53 mg; Mustaka (Cyperus rotundus L.) 42 mg; Satavari (Asparagus racemosus Willd.) 21 mg; Kulattha (Dolichos biflorus L.) 21 mg; Ushira (Vetiveria zizanioides (L.) Nash) 21 mg; Karchura (Curcuma zedoaria Roxb.) 14 mg; Trikatu 14 mg; Saindhava 50 mg; Suvanchika 42.5 mg; Yavakshara 5 mg; and Narasara 2.5 mg [9].

2.2 Animals

Twenty-four Wistar albino rats (180–200 g body weight) were used in this study. Animals were procured from the Institutional animal house (Reg no. 621/02/ac/ CPCSEA) of Birla Institute of Technology, Mesra, Ranchi. All animals were kept in standard housing conditions (room temperature 24–27 °C and humidity 60%-65% with 12 : 12 light:dark cycles). Food was provided in the form of dry pellets (Amrut Laboratory Animal feed, Maharashtra, India) and water ad libitum. All experiments involving animals complied with the ethical standards of animal handling and were approved by the institutional animal ethics committee.

2.3 Induction of urolithiasis

The EG-induced hyperoxaluria model of Atmani et al (2003) was used to induce urolithiasis in rats [10]. Rats weighing about 180–200 g were housed in metabolic cages three days prior to the start of the experiment for acclimatization. Animals were divided into four groups comprising six animals each: group 1 (naive); group 2 (control/untreated); group 3 (treated at 500 mg·kg⁻¹); group 4 (treated at 750 mg·kg⁻¹). EG was added in the drinking water for 28 days to induce a chronic, low grade hyperoxaluria and precipitate CaOx deposition in the rat kidneys. Group 1 (naive) was given water only and other rats (groups 2, 3 and 4) were given 0.75% (V/V) EG for the induction of urolithiasis. Mitra et al (1998) reported that 500 mg·kg⁻¹ body weight p.o. of Cystone is the minimum dose required for eliciting an optimal activity [11]. Consequently, 500 mg and 750 mg·kg⁻¹ body weight doses were selected for the experiment. The following simultaneous treatment of normal saline and Cystone were given orally (once a day) for 28 days to the respective groups.

Group 1 rats were given normal saline 10 mL·kg⁻¹ body weight, and served as the naive group; Group 2 rats were given normal saline 10 mL·kg⁻¹ body weight, and served as the hyperoxaluric-untreated (control) group; Group 3 rats were given Cystone at 500 mg·kg⁻¹ body weight, and served as a hyperoxaluric-treated group; Group 4 rats were given Cystone at 750 mg·kg⁻¹ body weight, and served as a hyperoxaluric-treated group.

During the 28 days of the study, body weight, water intake and animal health were observed regularly, so that stressed and unhealthy animals could be excluded from the study. Various biochemical parameters of urine and plasma were determined during the study. At the end the experimental period, animals were sacrificed by cervical dislocation and dissected to isolate kidneys for estimation of antioxidant markers and for histopathological analysis.

2.4 Urine analysis

Animals were kept in metabolic cages individually for the collection of 24 h urine samples on days 0, 7, 14, 21 and 28, and urine volume was measured immediately after collection, and also analyzed for calcium, phosphate, and citrate (Crest Biosystem Pvt. Ltd., India) and for oxalate (Sigma, USA) content using commercial kits. A semi-quantitative microscopic crystalluria analysis was performed by observing the number and size of crystals. Twenty-four hour urine samples were first mixed well and then aliquots were withdrawn and put on a slide and examined microscopically (Leica DME) [12].

2.5 Plasma analysis

After the experimental period, blood was collected by cardiac puncture after cervical dislocation. Plasma was separated by centrifugation at 10 000 × g for 10 min, than plasma creatinine and blood urea nitrogen (BUN) were estimated using commercial kits (Crest Biosystem Pvt. Ltd., India) [13].

2.6 Histopathological examination

After dissection, the isolated kidneys were cleaned of extraneous tissue and the weight recorded. Left kidneys were fixed in 10% formalin solution in 0.1 mol·L⁻¹ phosphate buffer saline and then dehydrated in ascending grades of alcohol and embedded in paraffin. Sections of 6 μm thickness were cut using a microtome, stained with hematoxylin and
eosin and examined under a light microscope (Leica EZ-4D) for histopathological changes [13].

2.7 Antioxidant marker estimation

The right kidney was used for the preparation of homogenate, and the separated renal cortex was kept at 4 °C and homogenized in cold potassium phosphate buffer (0.05 mol·L\(^{-1}\), pH 7.4). The renal cortical homogenates were centrifuged at 1500 × g for 10 min at 4 °C [13]. The resulting supernatant was used for the determination of various antioxidant enzymes and markers.

Malondialdehyde (MDA) was estimated by the method of Esterbauer and Cheeseman (1990), in terms of thiobarbituric acid reactive species [14]. The catalase (CAT) activity was measured using the method of Chance and Maehly (1955), following the decomposition of H\(_2\)O\(_2\) [15]. Reduced glutathione (GSH) was measured by the method of Dringen and Hamprecht (1996) using 5, 5'-dithiobis (2-nitrobenzoic acid) as a substrate [17]. Glutathione peroxidase (GPx) activity was measured by the method of Paglia and Valentine (1969) [18]. The protein carbonyl content was estimated by protein derivatization with dinitrophenyl hydrazine (DNPH) into chromophoric dinitrophenyl hydrazones by the method of Levine et al (1990) [19].

2.8 Statistical calculations

All the data expressed are mean ± SEM. All statistical comparisons between the groups were made by means of one-way analysis of variance (ANOVA), followed by post hoc "Tukey's Multiple Comparison Test" using Graphpad Prism 5 software. The \(P\) value less than 0.01 is regarded as significant.

3 Results

The urinary output was increased significantly (\(P < 0.001\)) in untreated, as well as Cystone-treated (\(P < 0.001\)) hyperoxaluric rats. It remained significantly (\(P < 0.01; P < 0.001\)) higher in Cystone-treated rats at both the 500 and 750 mg·kg\(^{-1}\) body weight, respectively, as compared to the untreated hyperoxaluric group (Table 1). An increased oxalate (\(P < 0.001\)), calcium (\(P < 0.01\)), and phosphate, and a decreased citrate excretion was observed in the hyperoxaluric rats (Group 2). However, supplementation with Cystone significantly prevented these changes dose-dependently in Groups 3–6 (Fig. 1). Microscopic urinalysis revealed that the control rats were devoid of crystals. However, untreated hyperoxaluric rats showed numerous aggregated CaOx crystals in the urine. Cystone therapy visibly reduced both the size and number of the crystals in a dose-dependent manner in the treated rats.

### Table 1 Parameters recorded for the assessment of anti-urolithiatic activity of Cystone in rats during 28 days (mean ± SEM, \(n = 6\))

<table>
<thead>
<tr>
<th>Observational parameter</th>
<th>Control</th>
<th>Untreated</th>
<th>Cystone, (500 \text{ mg·kg}^{-1})</th>
<th>Cystone, (750 \text{ mg·kg}^{-1})</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>24-HOUR URINE PARAMETERS</strong></td>
<td></td>
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<tr>
<td>Urine Volume (mL/24 h)</td>
<td>9.2 ± 0.43</td>
<td>14.3 ± 0.56(^a)</td>
<td>32.4 ± 1.76(^c)</td>
<td>38.7 ± 1.98(^c)</td>
</tr>
<tr>
<td>Urine pH</td>
<td>6.9 ± 0.02</td>
<td>6.1 ± 0.03(^a)</td>
<td>7.1 ± 0.06(^c)</td>
<td>7.3 ± 0.08(^c)</td>
</tr>
<tr>
<td>Crystalluria</td>
<td>–</td>
<td>+++</td>
<td>++</td>
<td>+</td>
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<tr>
<td><strong>PLASMA PARAMETERS FOR RENAL FUNCTION</strong></td>
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<tr>
<td>BUN (mg/dl)</td>
<td>24.80 ± 1.30</td>
<td>38.41 ± 1.82(^a)</td>
<td>28.16 ± 0.95(^c)</td>
<td>25.39 ± 1.18(^c)</td>
</tr>
<tr>
<td>Creatinine (mg/dl)</td>
<td>0.92 ± 0.08</td>
<td>1.64 ± 0.12(^a)</td>
<td>1.02 ± 0.06(^d)</td>
<td>0.94 ± 0.08(^c)</td>
</tr>
<tr>
<td><strong>KIDNEY PARAMETERS</strong></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Kidney Weight (g)</td>
<td>0.74 ± 0.02</td>
<td>1.2 ± 0.03(^a)</td>
<td>0.78 ± 0.04(^d)</td>
<td>0.76 ± 0.03(^c)</td>
</tr>
<tr>
<td>Crystal Deposits</td>
<td>–</td>
<td>+++</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td><strong>ANTIOXIDANT ENZYME/MARKER OF OXIDATION IN RENAL TISSUE</strong></td>
<td></td>
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<tr>
<td>Malondialdehyde (MDA) (nmol/mg protein)</td>
<td>668 ± 21.60</td>
<td>937 ± 23.10(^a)</td>
<td>787 ± 34.60(^d)</td>
<td>685 ± 18.20(^c)</td>
</tr>
<tr>
<td>Catalase (\mu\text{mol H}_2\text{O}_2) degrade/min</td>
<td>68 ± 2.10</td>
<td>39 ± 3.20(^a)</td>
<td>61 ± 4.20(^d)</td>
<td>67 ± 5.70(^c)</td>
</tr>
<tr>
<td>Super Oxide Dismutase (U/mg protein)</td>
<td>1.83 ± 0.02</td>
<td>0.95 ± 0.06(^a)</td>
<td>1.45 ± 0.13(^d)</td>
<td>1.78 ± 0.07(^c)</td>
</tr>
<tr>
<td>Reduced Glutathione ((\mu\text{mol/mg protein}))</td>
<td>12.10 ± 0.75</td>
<td>7.35 ± 0.91(^a)</td>
<td>10.64 ± 0.24(^d)</td>
<td>11.8 ± 0.52(^c)</td>
</tr>
<tr>
<td>Glutathione Peroxidase (U/mg protein)</td>
<td>18.2 ± 1.41</td>
<td>12.141 ± 0.85(^a)</td>
<td>17.8 ± 0.68(^d)</td>
<td>19.50 ± 1.15(^c)</td>
</tr>
<tr>
<td>Protein Carbonyl Content (mg/dl)</td>
<td>245 ± 10.2</td>
<td>314 ± 4.6(^a)</td>
<td>264 ± 8.0(^d)</td>
<td>220 ± 7.6(^c)</td>
</tr>
</tbody>
</table>

\(^aP < 0.001\) and \(^bP < 0.01\) vs the control group; \(^cP < 0.001\) and \(^dP < 0.01\) vs the untreated group
Hyperoxaluric treatment caused impairment of renal function in untreated hyperoxaluric rats as evident from the markers of glomerular and tubular damage, namely: raised BUN ($P < 0.001$) and plasma creatinine ($P < 0.001$), which were prevented in animals receiving simultaneous treatment with Cystone (Table 1).

Hyperoxaluria induction treatment enhanced MDA and total protein content ($P < 0.001$), decreased GSH level ($P < 0.001$) and the activities of the antioxidant enzymes, including SOD ($P < 0.001$), GPx ($P < 0.001$) and catalase ($P < 0.001$) in kidneys of the untreated rats as compared to the control animals. Simultaneous treatment with Cystone protected the animals against the oxidative changes induced by hyperoxaluric treatment in a dose-dependent manner (Table 1).

Kidneys excised from the untreated group were larger and heavier than those of the control animals ($P < 0.001$), When observed microscopically, many crystalline deposits were seen in the tubules of all regions of the kidneys, with marked dilation and widespread necrosis of the tubular epithelium (Fig. 2b). Whereas in the Cystone-treated group, kidney size and weight were normal, and crystal deposits were visibly small and rare, with decreased renal cell injury and normalization of renal architecture (Figs. 2c and 2d).

**Discussion**

The most harmful consequence of hyperoxaluria is deposition of CaOx crystals in the kidney. Cystone, a popular polyherbal formulation, was reported to be effective in urolithiasis \[11\]. In the present study, the beneficial antioxidant effect of Cystone against hyperoxaluric oxidative stress was evaluated. EG administration increased oxalate excretion significantly in hyperoxaluric animals as compared to control (Fig. 1). EG interferes with oxalate metabolism by way of increasing substrate availability which increases the activity of oxalate synthesizing enzymes. Glycolic acid oxidase (GAO) and lactate dehydrogenase (LDH) catalyse the oxidation and reduction of glyoxalate which results in the formation of glycolate and oxalate \[20\]. Simultaneous treatment with Cystone significantly lowered oxalate excretion, which may be due to the inhibitory action on GAO \[11\]. Cystone-treated animals showed increased urine volume with respect to naive and untreated animals. Increased palatability, due to the sweetness of EG, increased the water intake in untreated rats, followed by an increase in urine volume. It remained significantly higher in the Cystone-treated group \[13\]. Increase in urine volume in the treated rats was due to the diuretic effect of Cystone. Increased urine volume decreases the oxalate concentration and improves the supersaturation and crystallization, and prevents the precipitation of the CaOx in kidney. It also reduces oxalate toxicity to exposed renal epithelial cells and protects these cells from oxidative stress-induced injury. Diuresis also increases the amount of fluid going through the kidneys and provides mechanical expulsion of the deposits \[21\].

Calcium and phosphorus play a vital role in renal formation. Calcium and phosphorus levels were also elevated in the rats receiving EG (Fig. 1). The increase in calcium excretion may be due to defective tubular reabsorption in the kidneys \[22-23\]. Cystone treatment reduced the levels of calcium and phosphorus, and increased citrate excretion in urine. Citrate chelates the calcium ion preferentially compared with oxalate to form a soluble salt and inhibits nucleation. Microscopic observation revealed that untreated rats excreted abundant crystals, whereas Cystone treatment visibly reduced
the crystal size with a significant decrease in the number of crystals. Organic inhibitory compounds (e.g. glycosaminoglycans and other macromolecules) present in medicinal plant formulation may be adsorbed to the surface of the crystal, thereby inhibiting crystal growth and aggregation [24].

Plasma analysis showed that EG caused deterioration of renal function (raised BUN and plasma creatinine) due to retention of CaOx crystal agglomerates in renal tubules, which was improved by Cystone therapy. Extensive CaOx crystal deposition in the kidneys of untreated rats accompanied by oxidative damage, as reflected by increased levels of selected markers of oxidative injury: MDA and protein carbonyl content, and decreased activities of antioxidant enzymes, along with GSH level. Exposure to oxalate generates toxic responses in renal epithelial cells, including altered membrane surface properties, disruption of mitochondrial function, and the formation of ROS [3]. Several in vivo and in vitro studies have demonstrated that exposure to high levels of oxalate induced ROS, leading to redox imbalance, which is manifested as antioxidant depletion, lipid peroxidation and oxidation of protein [25-26]. Recent studies have provided evidence that kidney stone patients excrete significantly higher amounts of GST and MDA in their urine [27-28], with a concomitant decrease in the antioxidant enzymes SOD, CAT, and GPx, as well as GSH and protein thiols, indicating ROS in the kidneys of CaOx stone patients [6, 28]. Recent studies also demonstrated that vitamin E therapy prevents CaOx deposition in the rat kidney, and reduces renal cell injury by restoring these enzymes [29]. Similarly, antioxidant constituents (flavonoids) of Cystone effectively scavenge the free radicals and ROS, and protect the renal cell from oxidative stress-induced injuries, which is evident from the restoration of SOD, CAT, GPx, GST and GSH levels in Cystone-treated animals as compared to hyperoxaluric animals. Renal histopathological data also support the above results, as evident from the CaOx crystal deposition and tubular damage to the kidneys of untreated rats. Tissue injury, loss of membrane integrity and inflammation in the kidneys of these animals is due to hyperoxaluria-induced lipid peroxidation and depletion of antioxidant enzymes [29-31]. Renal epithelial injury promotes crystal adherence and retention, as epithelial injury exposes a variety of crystal adhesion molecules on epithelial surfaces [32]. However, treatment with Cystone inhibited crystal deposition and protected from renal injury (Figs. 2c and 2d). Thus, through the reduction of oxalate concentration, increased urine volume and restoration of the antioxidant enzymes, the Cystone protects renal cells against hyperoxaluria-induced oxidative stress.

This study revealed, for the first time, the protective antioxidant action of Cystone in hyperoxaluria-induced urolithiasis, besides the diuretic and urinary chemistry effects. However, it lacks an effect on oxalate metabolism, which is a further matter of investigation.

6 Conclusions

The results of this study indicate that Cystone inhibited lipid peroxidation, restored the antioxidant enzyme activity and prevented the CaOx crystal deposition in rat kidneys. Thus, various phytochemical constituents of Cystone synergistically combat hyperoxaluria-induced oxidative stress and prevent calcium oxalate crystal deposition in urolithiasis, possibly mediated through antioxidant and diuretic activity.

References


