

Research Article



Citrus Bioflavonoids Ameliorate Hyperoxaluria Induced Renal Injury and Calcium Oxalate Crystal Deposition in Wistar Rats

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Article info

Article History:

Received: 2 January 2015
Revised: 12 May 2015
Accepted: 14 May 2015
ePublished: 19 September 2015

Keywords:

- Citrus bioflavonoids
- Urolithiasis
- Hyperoxaluria
- THP
- Inflammatory marker

Abstract

Purpose: Citrus is considered as a medically important plant from ancient times and the bioflavonoids of different variety of citrus fruits were well explored for their biological activities. The study aim was to explore the effect of citrus bioflavonoids (CB) to prevent and cure hyperoxaluria induced urolithiasis.

Methods: Twenty four Wistar rats were segregated into 4 Groups. Group 1: Control; Group 2: Urolithic (EG-0.75%); Group 3: Preventive study (EG+CB, day 1-50); Group 4: Curative study (EG+CB, day 30-50). Animals received CB orally (20mg/kg body weight) after performing a toxicity study.

Results: Urinary risk factors and serum renal function parameters were significantly reduced by CB administration in both preventive and curative study ($p < 0.001$). Hematoxylin & Eosin and von Kossa staining demonstrated that renal protection was offered by CB against EG insult. Immunohistochemical analyses revealed over expression and abnormal localization of THP and NF- κ B in urolithic rats, while it was effectively regulated by CB supplementation.

Conclusion: CB prevented and significantly controlled lithogenic factors and CaOx deposition in rats. We propose CB as a potential therapy in management of urolithiasis.

Introduction

Urolithiasis refers to formation of stones in urinary tract, predominantly calcium oxalate (CaOx) stones. Various inorganic and organic promoters of CaOx crystal aggregation were reported like oxalate, uric acid, calcium and phosphate, while inhibitors comprise of the urinary macromolecules like Tamm-Horsfall protein (THP), Osteopontin, Prothrombin fragment 1, and other urinary macromolecules in addition to citrate and magnesium.¹ Though hyperoxaluria and CaOx crystal aggregation are major processes in stone formation, crystal adhesion to the damage epithelium is an important step in urolithiasis, which actually drives the disease to an advanced stage.² Oxidative stress caused by CaOx crystals and high oxalate load, leads to interstitial inflammation that can be marked by increased expression of various pro-inflammatory cytokines like, NF- κ B, p38-MAPK, etc.³ Dietary supplements and edible plant products are acclaimed for health benefits offered by their micronutrients and phytochemical ingredients.⁴ The diverse biological properties of herbal extracts attract medical experts and researchers to rely on plants for safer and prospective treatment for the kidney stone ailment.⁵ Significant curative effect of plants was due to various flavonoids and other antioxidant compounds present in it.⁶

Studies had shown that supplementation of antioxidants provide cellular protection to the kidney, by inhibiting the over expression of inflammatory cytokines and improved the antioxidant status of the whole system.^{7,8}

Citrus fruit and their peels consist of a wide range of flavonoid compounds including Eriocitrin, Hesperidin, Naringin, Naringenin, which are known for its abilities to offer beneficial biological properties.⁹ The major evident roles of citrus fruits in being a potential treatment for kidney stones were its ability to increase the urine pH and citrate content. Orange juice was the most favourable treatment that was reported from a number of clinical trials.^{10,11} The study conducted by Touhami et al., (2007) found that administration of lemon juice significantly reduced the incidence of CaOx crystal deposition in the kidneys of experimental rats.¹² Citrus is one of the genuine choices for treatment of kidney stones, since they are one of the richest sources of polyphenolic agents, organic and inorganic enrichments.¹³ Hence, in the present study we aimed to explore the preventive and curative effect of the commercially formulated citrus bioflavonoids in the management of hyperoxaluric animals progressing to calcium oxalate stone formation. We have also checked the expression and localization of THP in diseased and CB

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supplemented animals to exhibit the effective inhibition of crystal aggregation process by the flavonoids, which was primarily regulated by this anionic glycoprotein. On the other hand expression study of NF- κ B, a proinflammatory cytokine also elaborates the role of CB in handling the renal oxidative and inflammatory insult.

Materials and Methods

Animals

Wistar rats of 8 weeks, weighing 200 – 250 g were used for the study. All animal experiments and maintenance were carried out according to the ethical guidelines suggested by the Institutional Animal Ethics Committee (Registration No – 1333/c/10/CPCSEA). Animals were housed in polypropylene cages and maintained under standard conditions of 12 hours dark/light cycle at 27 \pm 1°C. The rats were supplied with regular pellets and water *ad libitum*.

Acute oral toxicity

CB tablets were purchased from Health Aid Ltd, UK, and the composition of CB is given in Table 1. Citrus bioflavonoids tablet was orally administered to the female Wistar rats and the safe dose was calculated according to the OECD guidelines 423.¹⁴ Animals were supplemented with CB at a dosage of 5000 mg/kg body weight. After administration the animals were monitored for 2 days to observe any immediate toxic effects. At the end of 14th day, the animals were sacrificed and blood was collected by cervical decapitation under anaesthetic condition. Blood collected with anticoagulant was used for haematological analyses while, blood without anticoagulant was centrifuged at 4000 rpm in 4°C to separate the serum for biochemical analyses.

Table 1. Composition of Citrus Bioflavonoids (CB) tablets

S. No	Content	Compounds*
1	Citrus Bioflavonoids	Flavonoids obtained from Citrus fruits
2	Bulking Agent	di-Calcium Phosphate
3	Binding Agent	Acacia gum
4	Anti-Caking Agent	Stearic acid and magnesium Stearate

* All the content are added at EC Recommended Daily Allowance

Anti-urolithic study

The anti-urolithic study includes 24 male rats segregated into 4 groups, which were used to demonstrate the preventive and curative effect of citrus bioflavonoid (CB). Group 1 consisted of normal animals while animals from group 2, 3 & 4 were challenged with 0.75% ethylene glycol (EG) for 50 days. Group 3 & 4 received CB (20 mg/kg of bodyweight) through oral administration. Group 3 animals received CB from day 1 to 50, whereas administration of CB was started from day 30 for group 4 animals and continued till end of the study.

Urine biochemical analyses

At the end of every 10th day rats were housed in metabolic cages and urine sample was collected under acidified

conditions. The collected urine samples were centrifuged at 2,500 rpm (REMI, R24, India) for 5 min and the supernatant was used to estimate the amount of calcium, urea, creatinine and protein using commercially available kits. Oxalate was measured by the method of Hodgkinson & Williams (1972), phosphate by the method proposed by Goldenberg & Fernandez (1966).^{15,16} The urinary urea level was expressed in g/24 hr urine and all other values are expressed as mg/24 hr urine.

Serum analyses

Serum analyses were performed on 50th day. The rats were anaesthetized and blood was collected from the retro-orbital region centrifuged at 10,000 rpm for 10 min, and the serum was separated. The serum was analysed for the presence of calcium, urea, phosphate, creatinine and protein and they are expressed as mg/dL. Calcium, urea, creatinine and protein were estimated using commercially available kits and phosphorus was measured using method proposed by Goldenberg & Fernandez (1966).¹⁶

Histopathological examinations

At the end of 50 days, the animals were euthanized under mild anaesthesia to avoid pain and stress. Kidneys were removed carefully and washed with phosphate buffered saline (PBS). The kidneys were fixed in 10% neutral buffered formalin and the fixed tissues were processed and embedded in paraffin. Sections were cut at a thickness of 4 μ m using Leica RM 2126 microtome and mounted on slides. The slides were stained by Haematoxylin & Eosin (H&E) and von Kossa for histopathological analyses. The sections were then photographed under microscope (Olympus BX51; Olympus optical, Tokyo, Japan) at a magnification of x400. H & E staining was used to study the tubular damage and crystal deposition while Von Kossa staining was performed to observe the intratubular calcium deposits.

Immunohistochemical analyses

Paraffin section (4 μ m thick) were cut mounted on slides, dewaxed in xylene and rehydrated in graded alcohol. Endogenous peroxidase activity was blocked by incubation with 3% H₂O₂ for 15 min. Antigen retrieval was performed for NF- κ B by heating the sections at 95°C for 10 min, citrate buffer (10mM, pH 6). After washing with PBS containing 0.1% Tween 20, the slides were incubated overnight with primary antibody for THP (1:200 dilutions) and NF- κ B (1:200 dilutions) at 4°C. The immunoreactivity was performed using horseradish peroxidase conjugated with goat-anti-rabbit IgG antibody by incubating for 30 min at room temperature. The detection step was performed by treatment with 3, 3'-Diaminobenzidine (Dako) as chromogen. Slides were counter stained with Haematoxylin, rinsed with tap water, dehydrated, cleared in xylene and mounted. The sections were then photographed at a magnification of x400 (Olympus BX51; Olympus optical, Tokyo, Japan).

Statistical analysis

The data were analysed on Graph Pad Prism 5.01 software and expressed as mean \pm SD (n=6). Statistical analysis was performed by One-way ANOVA followed by Dunnett's test to compare the diseased and the CB supplemented groups. At the same time, the statistical difference between the normal and experimental animals in toxicity study was analysed by Un-paired *t*-TEST. The results were considered statistically significant, if $P < 0.05$.

Results

Acute oral toxicity

Animals were found to be normal and did not show any signs of toxicity for 14 days after administration of CB at a concentration of 5000 mg/kg body weight of animals. The serum and haematological parameters of the CB supplemented rats showed no significant changes when compared to the control rats (Table 2 & 3), except for serum triglycerides level and platelet count, which however is in normal physiological range. Hence, the results of the toxicity study clearly suggested that the LD₅₀ cut off must be greater than 5000 mg/kg of body weight of animals and according to the Globally Harmonized Classification System (GHS), the CB falls under the category 5 or unclassified category of LD₅₀ range. Hence, it is safer to use at a concentration below 5000mg/kg of body weight. Based on literature and the prescribed dosage from the company, 20 mg/kg body weight of CB was selected for kidney stone study.^{17,18}

Table 2. Serum parameters of CB supplemented and control rats

Serum Parameter	Control	Citrus bioflavonoids (5000 mg/kg bodyweight)
Glucose ¹	83.5 \pm 9	81.9 \pm 8.1 ^{NS}
Urea ¹	39.4 \pm 6.1	40.7 \pm 6.5 ^{NS}
Creatinine ¹	0.99 \pm 0.06	0.85 \pm 0.1 ^{NS}
Total Cholesterol ¹	73.23 \pm 2.5	75.49 \pm 3.9 ^{NS}
Triglycerides ¹	100 \pm 3.8	121.9 \pm 5.33 [*]
Calcium ¹	11.05 \pm 1.3	10.08 \pm 0.8 ^{NS}
Albumin ²	4.57 \pm 0.2	4.41 \pm 0.5 ^{NS}
Protein ²	7.79 \pm 0.4	7.55 \pm 0.8 ^{NS}
Alanine Transaminase ³	26.62 \pm 3.5	27.3 \pm 0.8 ^{NS}
Aspartate Transaminase ³	47.52 \pm 8.5	46.23 \pm 3.6 ^{NS}
Alkaline Phosphatase ³	48.89 \pm 16.3	47.39 \pm 7.1 ^{NS}

1 – mg/dL; 2 – g/dL; 3 – U/L. The values are expressed as mean \pm SD (n=4). The study was carried out according to the OECD guidelines. 1 – mg/dL; 2 – g/dL; 3 – U/L. The values are expressed as mean \pm SD (n=3). The results were statistically analysed by non-parametric student's *t*-test. The comparisons were made as 'Control Vs Citrus bioflavonoids supplemented rats'. *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$, NS – Not Significant.

Urinary lithogenic factors

Urinary parameters after administration of EG showed drastic changes as a consequence of calcium oxalate crystal deposition in the kidneys. In our study we analysed the urinary parameters on every 10th day to observe the

progression of hyperoxaluric state with prominent crystal deposition and to check the effect of CB.

The EG supplemented animals showed a significant elevation in both calcium and oxalate excretion relative to hyperoxaluric animals supplemented with CB (Figure 1a & 1b). The hyperoxaluric rats showed significant excretion of urinary calcium (4.3 mg/ 24 hr) compared to control rats (3 mg/ 24 hr), whereas the excretion of calcium was reduced significantly in hyperoxaluric rats supplemented with CB. Oxalate excretion was also found to be raised in 24 hr urine of hyperoxaluric rats (18 mg) when compared to the normal animals, which were excreting 2 mg/24 hr. CB was found to be equally good in preventing and curing the abnormal excretion of these two major risk factors of renal lithogenesis. CB supplemented to group 3 animals from day one had maintained calcium and oxalate excretion closer to normal and considerably interfered with the disease processes. The animals in group 4 had an initial phase of hyperoxaluria and crystallization for a period of 30 days and administration of CB at such an advanced stage of the disease was also observed to be stabilizing the level of both calcium and oxalate.

Inorganic phosphate is another important constituent in urine, which can lead to progression of urolithiasis when its excretion rate is beyond the renal threshold. EG supplementation in group 2, 3 & 4 resulted in raised urinary phosphate level. Hyperoxaluric rats (Group 2) showed the highest phosphate excretion in urine at the end of 50 days (7 mg/ 24 hr). Reduced level of urinary phosphate excretion was observed in animals supplemented with CB from the beginning of the study while, the phosphate level in 24 hr urine of group 4 animals showed an increase till day 30, since the supplementation of CB was not initiated. On the commencement of CB administration, we observed a significant reduction in the urinary inorganic phosphate excretion and at the end of the study it was maintained at 5.76 mg (Figure 1c).

Table 3. Haematological parameters of CB supplemented and control rats

Haematological Parameters	Control	Citrus bioflavonoids (5000 mg/kg bodyweight)
RBC ¹	6.39 \pm 0.1	6.46 \pm 0.05 ^{NS}
WBC ²	4.1 \pm 0.2	3.93 \pm 0.2 ^{NS}
Platelet ³	560 \pm 8.5	397 \pm 11.1 ^{***}
Hemoglobin ⁴	12.5 \pm 0.3	12.72 \pm 0.2 ^{NS}
Hematocrit ⁵	37.5 \pm 0.3	38 \pm 0.4 ^{NS}
Mean corpuscular Volume ⁶	58.7 \pm 0.1	58.9 \pm 0.1 ^{NS}
Mean platelet Volume ⁶	8.4 \pm 0.4	8.1 \pm 0.3 ^{NS}
Mean corpuscular Hemoglobin ⁷	19.5 \pm 0.2	19.6 \pm 0.08 ^{NS}
Mean corpuscular Hemoglobin concentration ⁴	33.3 \pm 0.1	36.5 \pm 1.4 ^{NS}

The study was carried out according to the OECD guidelines. 1 – $\times 10^{12}/\mu\text{l}$; 2 – $\times 10^3/\mu\text{l}$; 3 – $\times 10^9/\mu\text{l}$; 4 – g/dL; 5 – %; 6 – Femtolitre (fL); 7 – Picogram (pg). The values are expressed as mean \pm SD (n=3). The results were statistically analysed by unpaired student's *t*-test. The comparisons were made as 'Control Vs Citrus bioflavonoids supplemented rats'. *** $p < 0.001$, ** $p < 0.01$, * $P < 0.05$, NS – Not Significant.

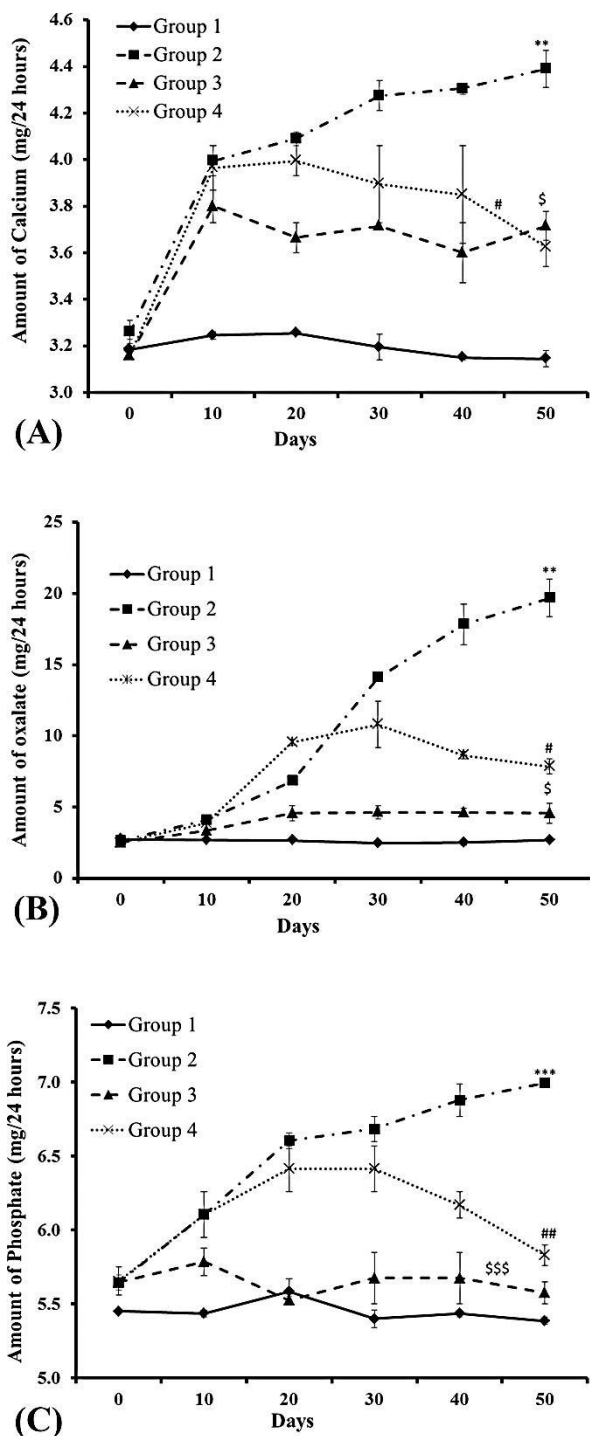


Figure 1. Effect of CB on urinary lithogenic factors (a) Calcium; (b) Oxalate & (c) Phosphate. Results are statistically analysed by one way ANOVA with Dunnett's multiple comparison post test (n=6). The comparisons are made as follows: '*' – Control Vs Diseased; '#' – Diseased Vs Prevention; '\$' – Diseased Vs Treatment. *** p<0.001, ** p<0.01, * P<0.05, NS – Not Significant.

Urinary renal damage markers

Urinary creatinine & urinary urea nitrogen are the scale of renal function in excretion of nitrogenous wastes from the system. EG challenged animals showed an abnormality in the excretion of creatinine and urea in 24

hr urine. The urolithic animals excreted a diminished amount of creatinine and urea of about 1 mg and 4 g /24 hr urine respectively (Figure 2a & 2b). CB was able to show protective effect on group 3 animals by alleviating these renal function parameters to near normal and it was also substantiated from the results obtained from group 4 animals, that the impaired renal function was also restored after supplementation of CB.

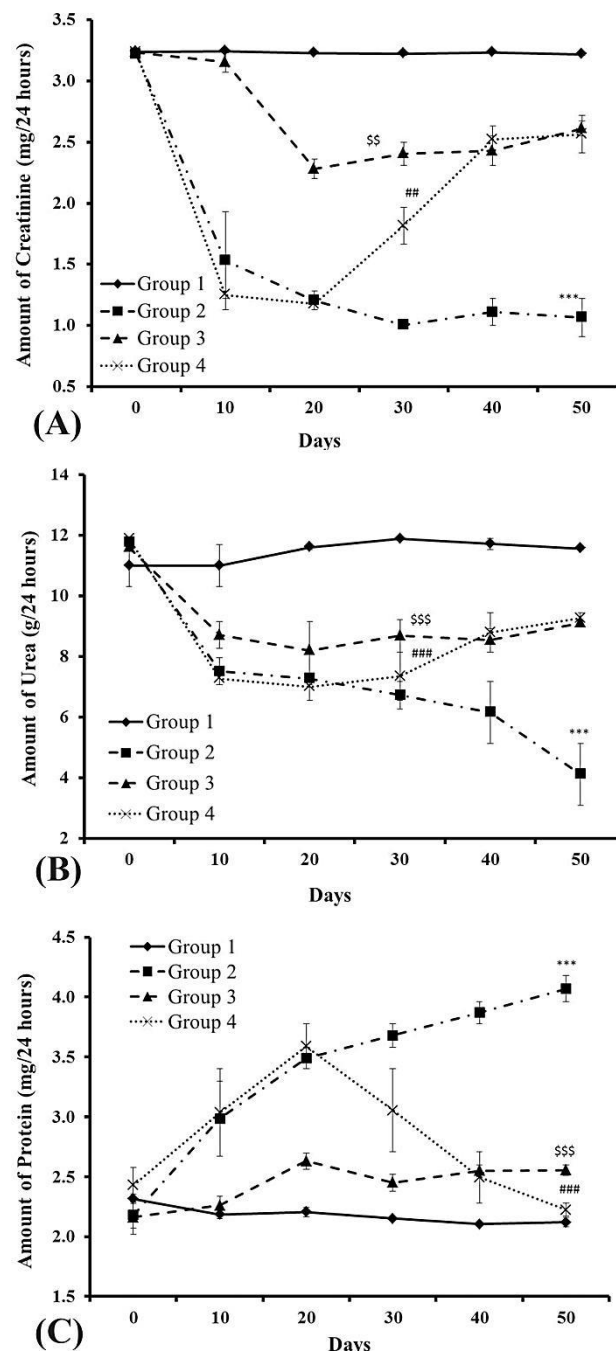


Figure 2. Effect of CB on urinary markers for renal damage (a) Creatinine; (b) Urea & (c) Protein. Results are statistically analysed by one way ANOVA with Dunnett's multiple comparison post-test (n=6). The comparisons are made as follows: '*' – Control Vs Diseased; '#' – Diseased Vs Prevention; '\$' – Diseased Vs Treatment. *** p<0.001, ** p<0.01, * P<0.05, NS – Not Significant.

Proteinuria, which was predominantly observed in the diseased animals, is a supportive observation of renal function. Citrus bioflavonoids had a constructive effect on protein excretion in both preventive and treatment strategies. As shown in the Figure 2c the excretion of protein in 24 hr urine of diseased animals showed as high as 4 mg, while CB supplemented animals had a check on excess protein excretion and maintained it close to the normal (2.5 mg/24 hr) excretion. This was observed in both group 3 & 4 animals, proving the ability of CB to protect and heal the damaged epithelium.

Serum parameters

Parameters like calcium, urea, creatinine and other lithogenic constituents were analysed in serum to show the effect of CB as an intervening substance in the genesis of CaOx stones and as a nephro-protectant. Serum parameters were analysed at the end of the study and the results are tabulated as shown (Table 4).

Increased urinary excretion of calcium and phosphate in EG supplemented rats had a reflection in the serum levels of these ions, which were correspondingly decreased. The results exhibit the positive effect of CB in sustaining the serum calcium & phosphate to 9.7 mg and 7.7 mg/ dL respectively (Table 4). Curative effect of CB also showed a significant observation in group 4 animals, where the initial reduction of calcium and phosphate in serum were restored close to normal.

The serum creatinine of EG challenged animals was found to be 2.9 mg/dL and blood urea nitrogen was 237 mg/dL (Table 4). Animals from group 3 were well protected from the CaOx and oxalate induced renal damage and this can be substantiated from the level of creatinine and blood urea nitrogen which was maintained at 1.8 mg and 82 mg in 100 ml of serum respectively. Whereas the accumulated nitrogenous wastes in serum of group 4 animals were cleared by the CB administration which started from day 30 and brought closer to normal level on 50th day (1.6 mg & 93 mg/dL). The reduction in the serum levels of urea and creatinine were concurrent to its increased urinary excretion.

Table 4. Effect of Citrus bioflavonoids on serum parameters on control and experimental groups

Parameter	Group 1	Group 2	Group 3	Group 4
Calcium	10.1±0.14	8.39±0.41 ^{a***}	9.7±0.25 ^{b*}	9.37±0.43 ^{ns}
Phosphorus	8.04±0.44	6.30±0.1 ^{a***}	7.76±0.1 ^{b*}	7.52±0.41 ^{c*}
Urea	91.9±3.2	236.9±7.2 ^{a***}	82.6±1.6 ^{b***}	93.0±4.2 ^{c**}
Creatinine	1.41±0.28	2.9±0.1 ^{a***}	1.82±0.1 ^{b***}	1.59±0.15 ^{c**}
Protein	1.16±0.06	1.54±0.08 ^{a***}	1.18±0.06 ^{b***}	1.21±0.07 ^{c*}

All the parameters were expressed as mg/dL.

The values are expressed as mean ± SD of four animals and results are statistically analysed by one way ANOVA with Dunnett's multiple comparison post test (n=6). The comparisons are made as follows

'a' – Control Vs Diseased

'b' – Diseased Vs Prevention

'c' – Diseased Vs Treatment

*** p<0.001, ** p<0.01, * P<0.05, NS – Not Significant.

Serum protein was slightly increased up to 1.5 mg/dL in the urolithic animals as a reflection of hepatotoxicity induced by EG, which was also ably prevented by CB in group 3 which showed a normal protein content, while in group 4 animals had a serum protein content of 1.21 mg/dL which was significantly lower than the diseased rats (Table 4).

Histology and immunohistochemistry

Urolithic rats are generally prone to crystal deposition in kidney after 30 days of ingestion with EG. The kidney sections of normal and experimental animals subjected to H & E and von Kossa staining (Figure 3). Significant deposition of crystals accompanied with tubular necrosis was observed in the kidney sections of the diseased rats. On the 50th day of the study, the animals supplemented with CB showed a prominent improvement in the kidney architecture. Rats from the preventive group showed near normal histology, since CB provided a strong membrane protection property; as a result of this a marked reduction in tubular necrosis and only a mild interstitial oedema were documented. The treatment group had a profound effect in repairing hyperoxaluria induced membrane damage which in turn helps in the expulsion of the deposited crystals. Kidney sections of group 4 rats showed tubular regeneration and moderate oedema. von Kossa staining of the kidney sections show dark intratubular aggregates, which confirm the presence of calcium containing stones. Dark spots observed in group 2 animals as a result of calcium deposition, were significantly reduced in CB administered rats. This substantiates the effectiveness of CB as an inhibitor of crystal aggregation and deposition process.

Expression of Tamm-Horsfall protein observed in animals from group 2 was found to be about 50 % greater than that of the control animals. THP, a secretory protein from nephron has been predominantly localized in distal tubule, was occasionally expressed in glomerulus of diseased kidney sections (Figure 4). Preventive therapy with CB showed a near normal expression of this protein without any abnormal localization. In group 4 animals we have detected a minimal elevation in the expression of this protein in the distal tubules which support the curative effect of CB. Nuclear Factor-κB is an important marker of the inflammatory process, whose expression was evidently increased in the lithogenic rats, as a result of oxalate and calcium oxalate crystal induced renal damage. This protein was mainly localized in the proximal and distal tubular regions (Figure 4). CB served from day one led to very low expression of this pro-inflammatory cytokine in preventive therapy and observed to be near normal, while an increased expression of NF-κB compared to control was seen in Group 4 animals, but significantly low compared to diseased animals. This shows that the physiological and biochemical events in the renal environment were brought to normal by CB.

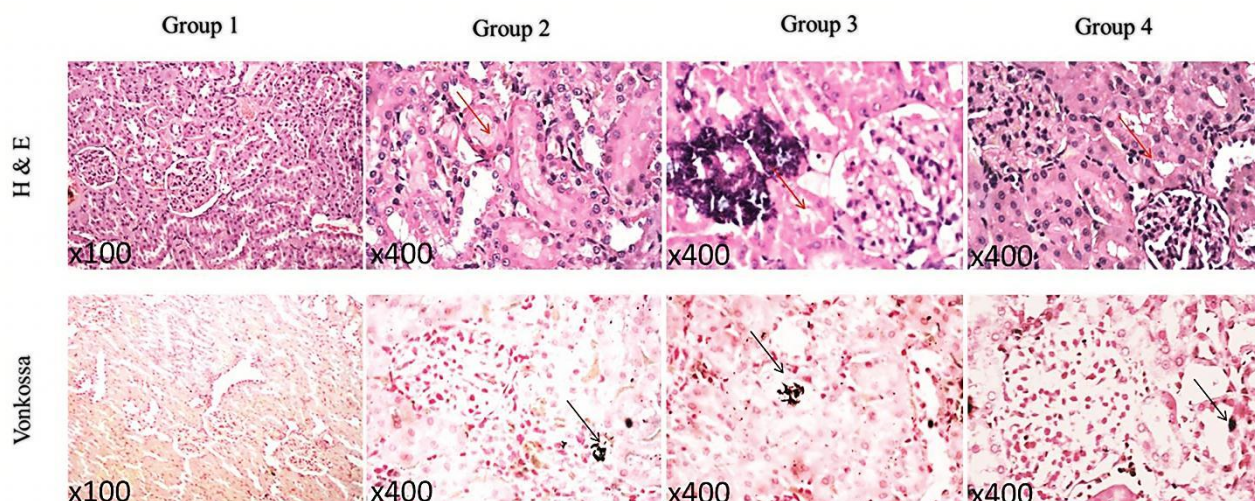


Figure 3. Histopathological staining of normal and experimental rats' kidney sections
Figure shows kidney sections of control and various experimental groups stained with Haematoxylin & Eosin and Von Kossa. Red arrows indicate the tubular changes and Black arrow shows the crystal deposition as brown spots

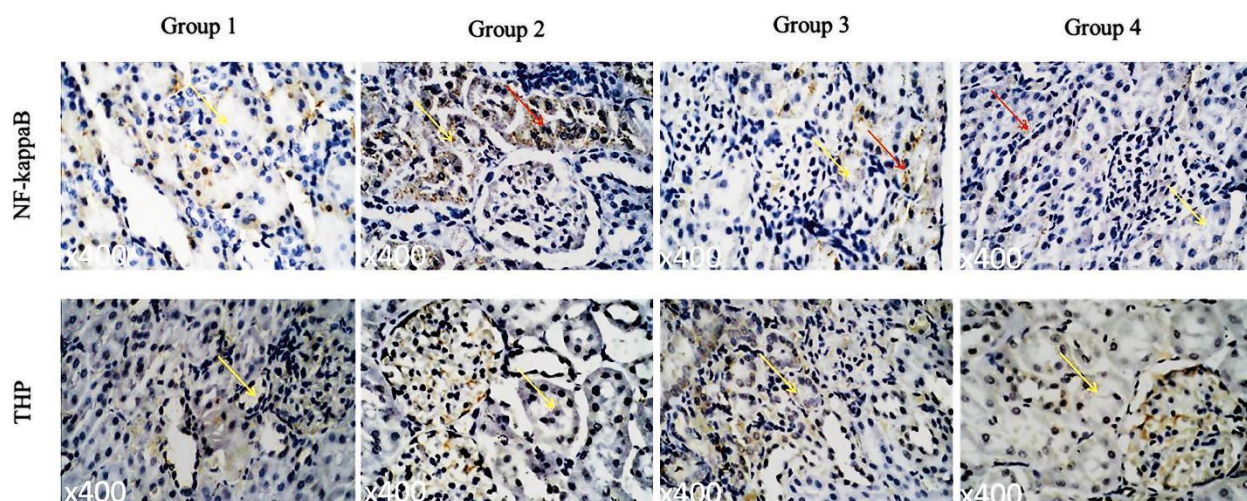


Figure 4. Immunohistochemical analyses of normal and experimental rats' kidney sections
Figure shows immunohistochemical expression of NF-kB and THP in control and various experimental groups. Red arrow indicate the proximal tubules and Yellow arrow indicate the distal tubules

Discussion

Herbal treatment in management of urolithiasis has become very popular among the researchers since it was found to be biocompatible and efficient. This can be solely attributed to the polyphenolic compounds present in those highly valuable plants and their parts which include flavonoids, alkaloids, triterpenes, and anthroquinones.¹⁹ Studies have shown that citrus flavonoid as an effective nephroprotective agent and it was found to exert significant anti-inflammatory and analgesic properties.^{20,21} In our investigation we proved that bioflavonoids obtained from this fruit also participate in the management of urolithiasis along with the indigenous citrate content. The study was designed in such a way to investigate the prophylactic and curative effect of citrus bioflavonoids. The CB formulation

contains the essential bulking, binding and anti-caking agents. The major ingredients of this tablet are the bioflavonoids (1000 mg) obtained from citrus fruits like, hesperidin, eriocitrin, naringenin, neohesperidin etc. These are the flavonoids that are well-known for its biological properties. Apart from the flavonoids, the flavones and flavonols derivatives of the flavonoids from the fruits are also supplied in the tablets. The bioflavonoids and its derivatives from citrus origin are found to be effective in prevention and treatment of various metabolic, degenerative and infectious diseases.⁵ Animals supplemented with EG showed first sign of the disease at the end of 20 days by excreting excess of oxalate and calcium, clinically represented as hyperoxaluria & hypercalciuria respectively. Increased oxalate metabolism in liver is responsible for

hyperoxaluric condition in EG supplemented animals, while hypercalciuria has a multiple aetiological origin such as membrane damage, increased absorption of dietary calcium etc.^{22,23} Our results evidently demonstrate the role of CB in maintaining the calcium and oxalate homeostasis, which can be an outcome of the renoprotective effect of bioflavonoids against the oxidative and nitrosative stress.²⁰ Heneghan et al., (2010) found that oxalate reabsorption is happening in the intestine through an anionic transporter mechanism, and we assume that this can also be ably targeted by the bioactive compounds in CB leading to dampen the increased rate of oxalate excretion in urine resulting in increased enteric excretion of oxalate.²⁴ Usually hypercalciuria will be accompanied by increased excretion of phosphorus in urine, designing a condition more favourable for stone formation and this condition was successfully reversed by CB administration in our study.²⁵ The observed urinary concentration of calcium and phosphorus coincides with the improvement in serum levels of the respective ions.

Reduction in glomerular filtration rate can be an outcome of crystals obstructing the renal passage.²⁶ This leads to accumulation of significant amount nitrogenous wastes like creatinine and urea. The obvious increase in serum creatinine and urea shows the renal dysfunction. This was well supported by the low level in the excretion of these nitrogenous wastes in urine of hyperoxaluric animals relative to normal animals. The flavonoids in commercial CB tablets were potent enough to maintain the normal renal function by protecting the membrane from crystal adhesion, which can lead to the obstruction anywhere in the urinary tract. Membrane protection offered by citrus flavonoids after supplementation, resulted in significant reduction of proteinuria compared to the EG administered animals which showed a high level of protein in urine as a result of oxidative stress exerted on the renal epithelium by calcium oxalate crystals and free oxalate. This can also be interpreted that the flavonoids can protect the renal cells by combating the ROS & RNS.²⁰

The serum and urine biochemistry showed a significant change due to induction of the hyperoxaluric condition and reversal of these abnormal values to normal was observed upon CB ingestion. This was well supported by the Hematoxylin and Eosin staining, where observed acute tubular damage due to calcium oxalate crystallization in EG supplemented rats was found to be protected and treated by CB. Calcium deposits observed as brown spots distinguish the calcium oxalate nephrolithiasis from other type of stones. EG supplemented rats showed a prominent calcium deposition and animals fed with CB were found to have significantly reduced or no deposits (Figure 3).

Elevated expression of NF- κ B was observed in kidney sections of hyperoxaluric animals as a result of inflammation mediated cellular damage due to EG challenge. Earlier studies were demonstrated that the inflammatory process in the epithelial cells was

organized by NF- κ B with the help of Renin – Angiotensin system (RAS) and researchers presume that inhibition of angiotensin converting enzyme (ACE) can diminish the calcium oxalate crystal mediated inflammation and epithelial degeneration.^{27,28} In our study suppression of this pro-inflammatory cytokine to normal level is a consequence of antioxidant and anti-inflammatory effect exerted by the citrus flavonoids.²⁹ The ACE inhibitory activity of the flavonoids (in this case citrus flavonoids) can also be associated with the reduction in inflammatory response.³⁰ THP is one of the abundant proteins found in the urine and it was reported to be one of the important crystal inhibitory proteins and the expression of this particular protein was increased as expected in the EG challenged animals in order to meet up with the amount of crystal formed.³¹ The reduction of this protein obviously shows the reduction in the crystal formation and deposition in the experimental animals supplemented with CB.

Conclusion

In conclusion, risk factors of stone disease were well managed by the administration of CB, which resulted in the cellular protection. Our results suggest bioflavonoids obtained from citrus fruits evade the urolithic risk factors like calcium, oxalate etc., and corresponding renal dysfunction (Urea, creatinine etc.). The major outcome in our study is the ability of CB to attenuate the expression of NF- κ B, which initiate the renal epithelial damage. This shows that, CB is not only as an anti-urolithic agent but, it can also act as an efficient nephroprotectant. This shows effect of bioflavonoids from citrus fruit could be effective for management of calcium oxalate nephrolithiasis and restoration of the impaired kidney to normal. Further studies on various higher animal models and effective clinical trials may lead to a novel formulation for the management of urolithiasis and other renal diseases.

Acknowledgments

Shiju TM and Badrinathan S are thankful to Council of Scientific and Industrial Research and Indian Council of Medical Research respectively for providing fund in the form of Senior Research Fellowship. Authors are also thankful to VIT University for providing instrumentation and other resources to carry out the project.

Ethical Issues

Not applicable.

Conflict of Interest

The authors declare that they have no conflict of interests.

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