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EFFECT OF METHANOLIC EXTRACT OF PEEL OF *PUNICA GRANATUM* ON ETHYLENE GLYCOL AND AMMONIUM CHLORIDE INDUCED NEPHROLITHIASIS

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ABSTRACT

The aim of this work is to evaluate the effect of *Punica granatum* on ethylene Glycol and ammonium chloride induced Nephrolithiasis by using male wistar albino rats. The rats were divided into 7 groups, which include both preventive and curative study. Renal calculi are been induced by the administration of 0.75% of Ethylene Glycol and 1% of Ammonium chloride for a period of 28days and 5days respectively. Two different doses i.e., 200mg/kg and 400mg/kg of the extract were been administered for 28days in preventive study and same doses of extract are administered from 14 to 28days in curative study. Parameters like oxalate, calcium, and phosphate in urine and creatinine, urea and uric acid in serum have been assessed. The histopathological studies of kidney and Phytochemical screening of the plant extract were also carried out. The levels of biochemical parameters were increased in ethylene Glycol and Ammonium chloride intoxicated rats when compared with the normal group. The extract, at doses of 200 and 400 mg/kg, exhibited significant ($p < 0.001$) reduction in biochemical parameters (urine: calcium, oxalate, phosphate& serum: BUN, urea, creatinine). Nephrolithiasis activity was also confirmed by histopathological findings. Furthermore, the phytochemical profile of the extract revealed the presence of alkaloid, phenols, flavanoids, steroids and triterpenoids. The results assure that *Punica granatum have been* empowered with antilithiatic activity which might be due to the presence of polyphenols.

Key words: *Punica granatum,* Ethylene Glycol, Ammonium chloride, Oxalate, Nephrolithiasis.

INTRODUCTION

Pomegranate (Punica granatum linn.,) a species of punicaceae, have grabbed the attention of scientists who engage themselves in Pharmacological, Pharmaceutical and Nutrological research due to its multiple bioactivities. Pomegranate (*Punica granatum*), an ancient fruit-bearing deciduous shrub, is the predominant member of two species comprising the *Punicaceae* family. It is a native of the Himalayas in northern India, but it has been cultivated and naturalized throughout the Middle East, the entire European Mediterranean region, the drier parts of Southeast Asia, northern and tropical Africa, and to some extent the United States, specifically California and Arizona (Viladomiu M *et al.,* 2013). The fruit and includes flavonoids (flavonols, flavanols, and anthocyanins), condensed tannins (pro-anthocyanidins) and hydrolysable tannins (HTs) (ellagi-tannins and gallo-tannins) (Seeram N *et al.,* 2005). These tannins are highly susceptible to both enzymatic and non-enzymatic hydrolysis. The hydrolysis products include glucose and ellagic acid or gallic acid. Additional phytochemicals present in pomegranate peel include organic and phenolic acids, sterols and triterpenoids, and alkaloids (Fischer U *et al.,* 2011). The ellagi-tannins present in the pomegranate peel accounts for approximately 92% of the total antioxidant activity of pomegranate fruit (Fischer U *et al.,* 2000). Therefore, the health benefits of pomegranate peel are accredited for the pharmacological activities exhibited by bioactive phytochemicals like polyphenols.

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The pomegranate was known to possess anti oxidant, anti diabetic, lipid regulator, anti inflammatory and anti neoplastic activities. The *anti oxidant activity* is been shown due to its free radical scavenging activity. It proved to possess the *anti neoplastic effect* by acting at 2 different stages the *apoptosis induction* and *proliferation invasion inhibition.* The extract had a minimal effect on normal cells and greater inhibiting effect on the tumour cells suggesting its potential therapeutic activity. The aqueous suspension of pericarp has shown to possess immunomodulation by stimulating both cell mediated and humoral mediated responses (Rufeng wang *et al.,* 2010).

Currently, open renal surgery for Nephrolithiasis is unusual and used only rarely since the introduction of Extra Corpeal Shockwave Lithotripsy (ESWL), which has revolutionized urological practice and almost become the standard procedure for eliminating kidney stones. However, in addition to the traumatic effects of the shockwave, persistent residual stone fragments, and the possibility of infection, suggests that ESWL may cause acute renal injury, a decrease in renal function and an increase in stone recurrence (Kishimo T *et al.,* 1986; Begun F.P *et al.,* 1991).

Recently obtained human data are also suggestive of the development of oxidative stress in hyperoxaluric kidney stone patients [Huang HS *et al.,* 2010]. Experiments have shown that supplementation of agents which could decrease oxidative stress was able to rescue the cells from oxalate-induced toxic effects. The peel of the fruit consists of greater % of antioxidant. So, in the present study an attempt has been made to establish the pharmacological activity of peel of *punica granatum*.

MATERIALS AND METHODS

Preparation of plant extract

Pomegranate peels were manually separated, shade dried and grounded to powder. The powder (25g) was extracted by mixing using a magnetic stirrer with 100 ml methanol at 30°C for 1 hr. The extract was filtered to remove the peel particles. The residue was reextracted with the same solvent. The extracts were pooled and concentrated under vaccum at 40°C (El-Sayed and El-Habibi, 2013).

Experimental animals

Colony inbred strains of S.D strain rats of male sex weighing 250-300g were used for the pharmacological studies. The animals were kept under standard conditions (day/night rhythm) 8.00 am to 8.00 p.m, 22 ± 1^0C room temperature, in polypropylene cages. The animals were feed on standard pellet diet and *water ad libitum.* The animals were housed for one week in polypropylene cages prior to the experiments to acclimatize to laboratory conditions. It was randomly distributed into four different groups with six animals in each group under identical conditions throughout the experiments.

The experimental protocol was approved by the Institutional Animal Ethical Committee (IAEC) of Vignan Institute of Pharmaceutical Technology approved by committee for the purpose of control and supervision of experiment on animals CPCSEA (Committee for the Purpose of Control and Supervision of Experimental Animals) with registration number 1499/Po/a/ 11/CPCSEA approved the study.

Acute oral toxicity studies

The procedure was followed by using OECD guidelines (organization of economic corporation and development) 423(acute toxic class method). One tenth of median lethal dose (LD_{50}) was taken as a effective dose (Anupama S and Handa SS, 1990).

Animal model

The male Wistar strain rats were randomized into 7 different groups (n=6 per group) in both preventive and curative study. Group I received food and water ad libitum. Groups II to VII received stone induction treatment for 28days, 0.75% ethylene Glycol and 1% ammonium chloride for 5 days (Samra Bashir, 2009). Group III served as a standard and received 750mg/kg of cystone for 28days. Groups IV & V served as preventive regimen, Group IV received MEPP 200mg/kg; Group V received MEPP 400mg/kg from 1 to 28^{th} day. Group VI &VII served as curative regimen, Group VI received MEPP 200mg/kg, and Group VII received MEPP 400mg/kg from 14^{th} to 28^{th} day. Stone induction is done by administering Ethylene Glycol for 28days and Ammonium chloride for 5days were added in drinking water for 28days. The extracts are administered through oral route one time a day.

Biochemical assays

On the 28^{th} day animals were housed in metabolic cages and collected urine for 24hrs and a drop of Hcl was added to the sample. Later the sample was subjected to analyze the calcium, oxalate, phosphorous. Blood samples are withdrawn from the retro orbital plexus to asses BUN, uric acid and creatinine in serum.

The parameters are assessed using semi auto analyser using standard methods.

Histopathololgical assays

At end of the 28^{th} day animals were sacrificed, kidneys were isolated and are sent for the histopathological studies.

The sections of kidney (cortex, medulla, and papilla) were stained with hematoxylin and eosin to study the histopathological changes and calcium oxalate crystal deposition.

Statistical analysis

The statistical analysis was carried out using Graph pad prism. All values were expressed as Mean ±S.E.M. Data analysis was done by one-way ANOVA followed by Tukey's test. Difference level at $P<0.05$ was considered as statistically significant condition.

RESULTS

Table 1. Effect of MEPP on urinary and serum parameters in control and experimental animals

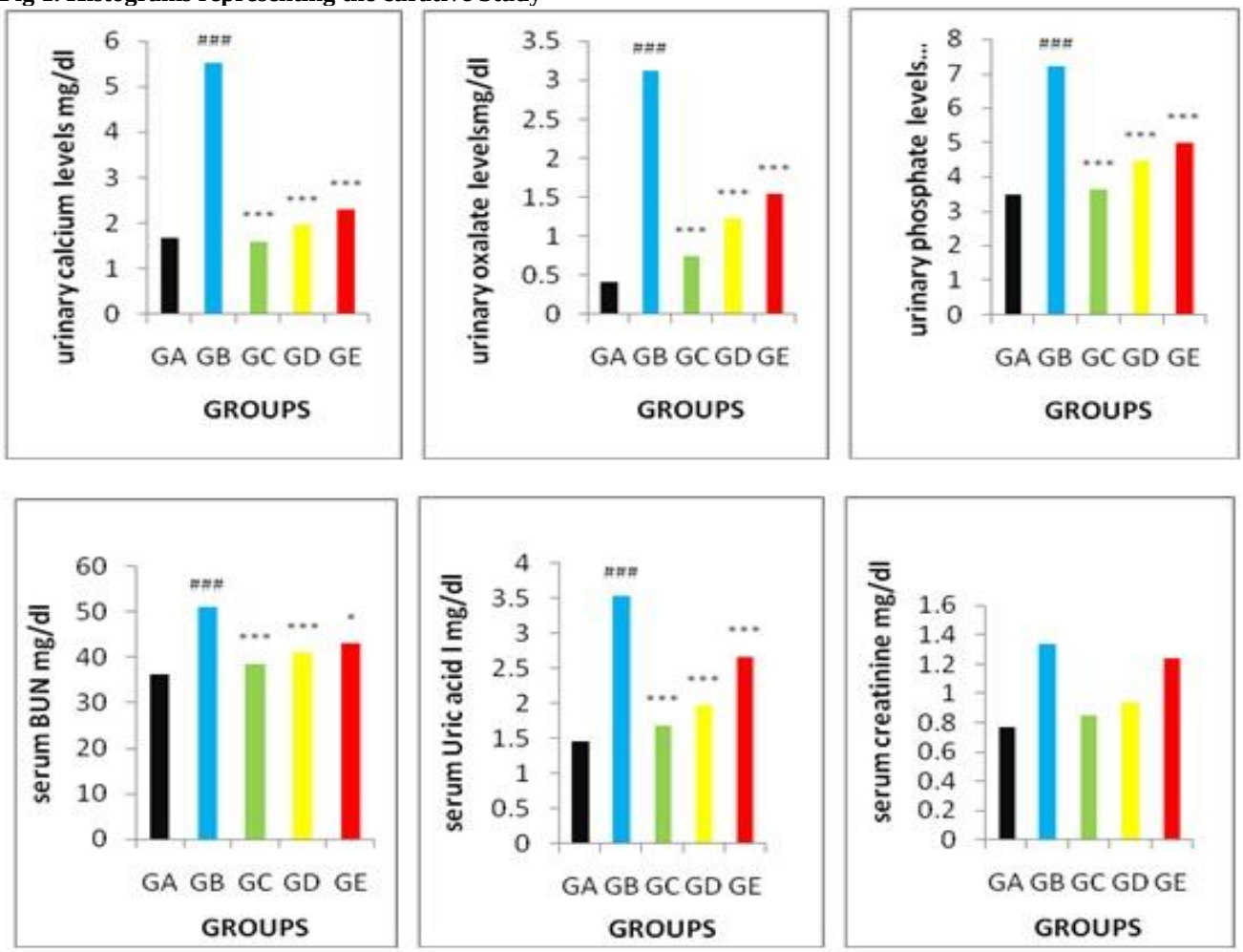
Parameter (unit)	Group I (Normal)	Group II (control)	Group III (standard)	Preventive Regimen		Curative Regimen	
				Group IV (200mg/kg)	Group V (400mg/kg)	Group VI (200mg/kg)	Group VII (400mg/kg)
Urine (mg/dl)							
Calcium	1.686 ±0.23	5.516 ±0.55$^{###}$	1.608 ±0.131***	2.163 ±0.370***	1.843 ±0.225***	2.34 ±0.162***	1.95 ±0.234***
Oxalate	0.423 ±0.044	3.121 ±0.205$^{###}$	0.756 ±0.069***	1.235 ±0.288***	0.816 ±0.099***	1.548±0.112***	1.235±0.089***
Phosphate	3.46 ±0.113	7.21 ±0.221$^{###}$	3.64 ±0.006***	5.1 ±0.358***	4.005 ±0.226***	5±0.090***	4.463±0.207***
Serum mg/dl)							
BUN	36.415 ±0.275	51.215±0.355$^{###}$	38.7 ±0.302***	45.411 ±2.76***	39.508 ±0.55***	43.21 ±0.44***	41.16 ±0.416*
Creatinine	0.745 ±0.029	1.346 ±0.082$^{###}$	0.853 ±0.018***	0.993 ±0.139***	0.938 ±0.106***	1.243 ±0.074***	0.945 ±0.007***
Uric acid	1.475 ±0.012	3.54 ±0.079$^{###}$	1.696 ±0.056***	2.305 ±0.293***	1.945 ±0.079***	2.67 ±0.152***	1.986 ±0.85***

Comparisons were made between the groups

Group II vs. Group I * Group III, IV, V, VI, VII vs. Group II.

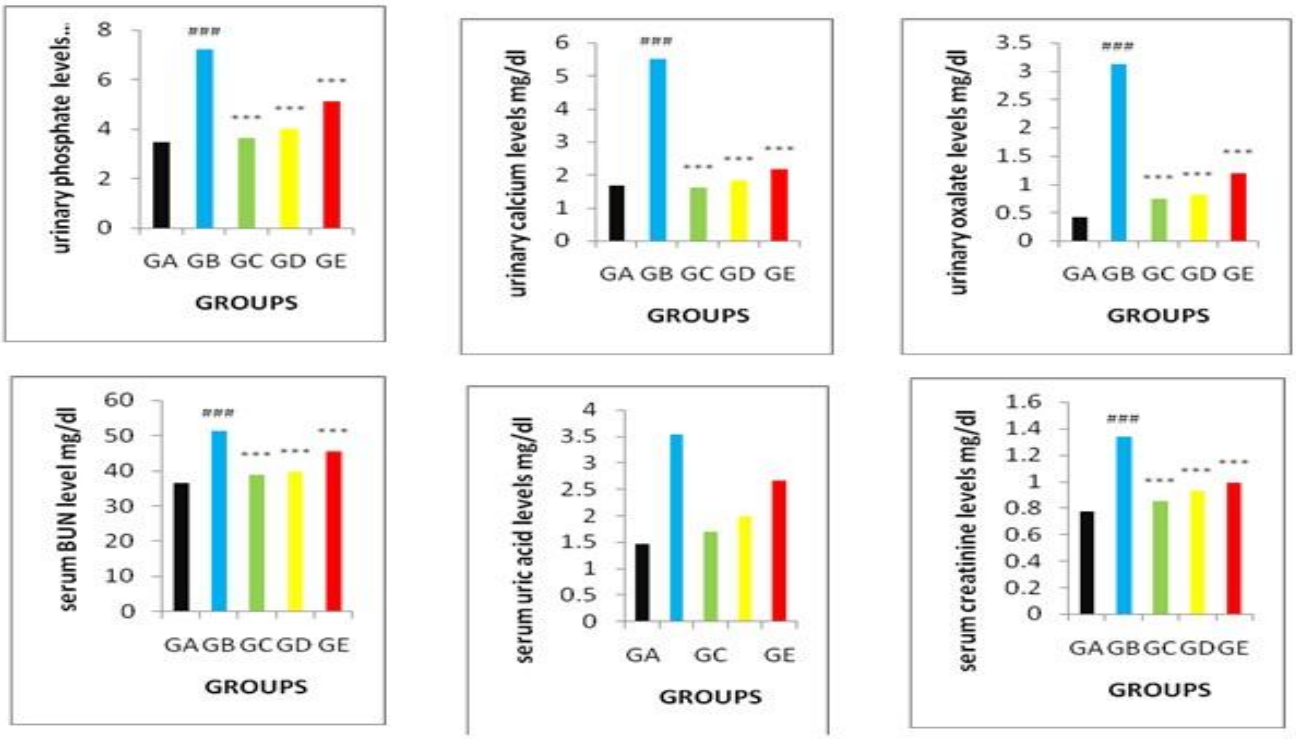
Symbols represent the statistical significance done by ANOVA one way test, followed by Tukey's multiple comparision test

Fig 1. Histograms representing the curative Study



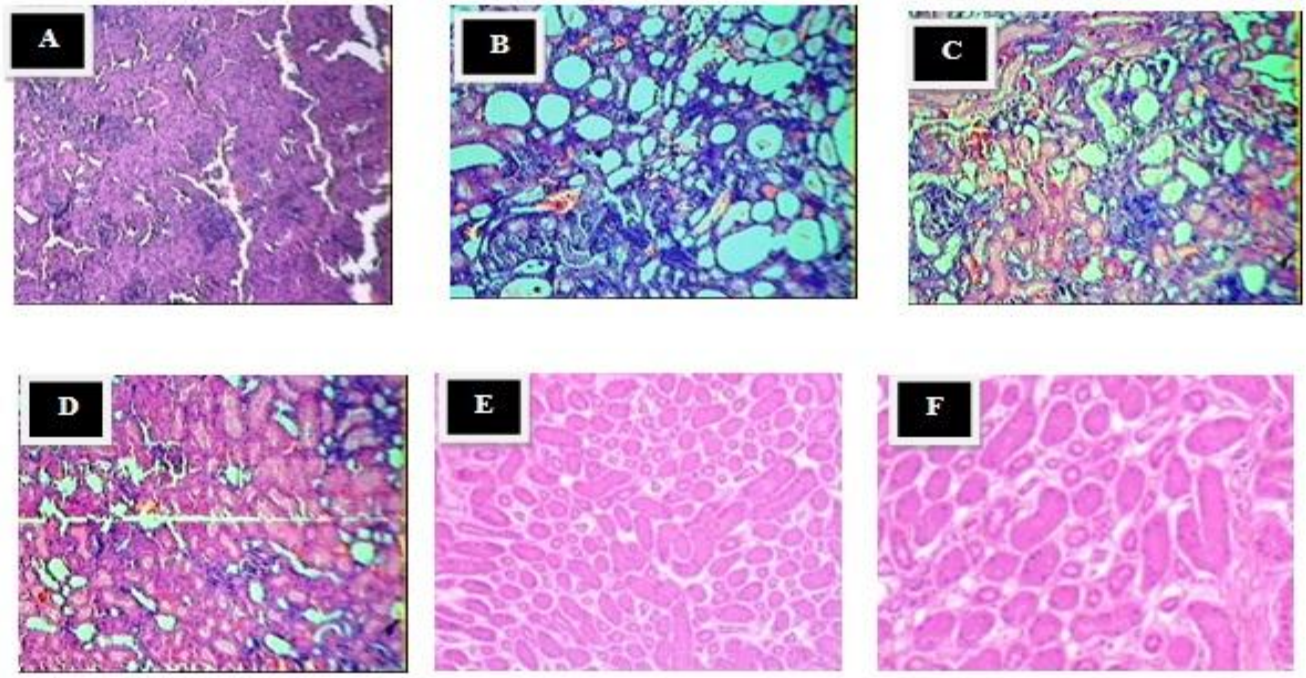
Group A - Normal; Group B – control; Group C- standard; Group D &E – Curative groups (200mg/kg and 400mg/kg respectively)

Fig 2. Histograms representing the preventive study



Group A - Normal; Group B – control; Group C- standard; Group D &E – Preventive groups (200mg/kg and 400mg/kg respectively)

Fig 3. Histopathological Studies



A – Normal; B – Control (0.75% ethylene glycol and 1% ammonium chloride); C & D (0.75% ethylene glycol and 1% NH_4Cl+ 200mg/kg, 400mg/kg MEPP (from 1-28days)); E&F (0.75% ethylene glycol and 1% NH_4Cl+ 200mg/kg, 400mg/kg MEPP (from 14-28days).

DISCUSSION

Urinary supersaturation with respect to stone-forming constituents is generally considered to be one of the causative factors in calculogenesis. Evidence in previous studies indicated that in response to 28 day period of ethylene glycol (0.75%, v/v) administration, young male rats form renal calculi composed mainly of calcium oxalate [Selvam *et al.,* 2001, Huang, H.S *et al.,* 2002]. The biochemical mechanisms for this process are related to an increase in the urinary concentration of oxalate. Stone formation in ethylene glycol feed animals is caused by hyperoxaluria, which causes increased renal retention and excretion of oxalate (Selvam *et al.,* 2001).

The study of the urinary chemistry with respect to the stone forming minerals will provide a good indication of the risk of stone formation. Hypercalciuria in ethylene glycol induced nephrolithic rats might be a factor favouring the nucleation and precipitation of calcium phosphate (apetite) from urine and subsequent crystal growth. In the present study, calcium excretion was increased in calculi induced animal (Group II). Treatment with MEPP reduced the level of calcium excretion in ethylene glycol and ammonium chloride induced nephrolithic rats.

Hyperoxaluria is a far more significant risk factor in the pathogenesis of renal stone than hypercalciuria. Urinary oxalate was increased in ethylene glycol induced urolithic rats, it has been reported that oxalate plays an important role in stone formation and has about 15-fold greater effect than urinary calcium (Borghi et *al.,* 1996). We observed an increased level of urinary oxalate in negative control group, which was significantly reduced in MEPP pretreated group. This indicates that its polyphenolic compounds undergoes some oxidative reactions and mainly act as inhibitors of some steps of oxalic acid synthesis from ethylene glycol.

An increase in urinary phosphate is observed in calculi induced rats (group II). Increased urinary phosphate excretion along with hyperoxaluria induced oxalate stress seems to provide an environment appropriate for stone formation by forming calcium phosphate crystals, which epitaxially induces calcium oxalate deposition (Roger *et al.,* 1997). Treatment of MEPP restores the urinary phosphate level thus decreases calcium oxalate formation.

In urolithiasis, due to stones in the urinary system obstruct the urine outflow. This leads to the accumulation of waste products in the blood, particularly nitrogenous substances such as urea, creatinine and uric acid (Ghodkar., 1994). 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 membranes and further promote the calcium oxalate deposition thereby decreasing the renal function. In the present study, the negative control calculi-induced rats (Group II) were found to have marked renal damage, consistent with the elevated serum levels of creatinine. The administration of MEPP inhibited this change that would otherwise promote new stone formation in the urinary system.

Uric acid is known to promote calcium oxalate crystal growth. The predominance of uric acid crystals in calcium oxalate stones and the observation that uric acid binding proteins are capable of binding to calcium oxalate and modulate its crystallization and also suggest its primary role in stone formation (Kalaiselvi *et al.,* 1999). In the present study, the concentration of uric acid level was increased in negative control group. MEPP treatment decreased the uric acid level in urine to normal level thus reducing the risk of stone formation.

Microscopic examination using polarized light of kidney sections derived from nephrolithiatic rats showed intratubular and interstitial crystal deposits, consistent with the findings of others (Grases *et al.,* 1998). Rats treated with MEPP had far less kidney calcification than the negative control rats (Group II). Reduction in papillary crystal deposition was seen in rats treated with MEPP. These results clearly demonstrate the ability of the MEPP to prevent the development of papillary calcifications on the kidney, consequently preventing the development of papillary calculi.

Hyperoxaluria and CaOx crystal formation are injurious to renal epithelial cells. These findings supported the hypothesis that apoptotic changes do occur during the hyperoxaluric phase, and that these alterations may result from free radical formation causing lipid peroxIdation. Apoptotic changes observed in renal tubular epithelial cells damaged by massive hyperoxaluria might result in cell degradation and could be responsible for the pathologic course of urolithiasis (Kemal Sarica *et al.,* 2004).

CONCLUSION

In conclusion oral administration of MEPP shows that *Punica granatum* protected and also cured the rats from ethylene glycol induced and ammonium chloride induced nephrolithiasis. The protective and curative effect of MEPP decreases the oxalate synthesis in the induced animals. These observations suggest MEPP is clinically protective against the oxidative stress induced by calcium oxalate deposition which might be due to the presence of polyphenolic antioxidants.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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