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Anatolian Propolis Prevents Oxalate Kidney Stones: Dramatic Reduction of Crystal Deposition in Ethylene-Glycol-Induced Rat Model

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Abstract: One of the many properties of propolis, a gift of nature, is that it is a potent antioxidant agent, which has been shown to be a miracle-worker in many different diseases. In this study, its possible protective and reversing effects against hiperoxaluria was investigated in a rat model in comparison with verapamil. In all 5 groups (Total n=76), aside from the control, hiperoxaluria was induced with continuous ethylene glycol (EG) administration. The others received EG only, 50 mg/kg propolis, 100 mg/kg propolis and 1mg/kg verapamil. To estimate the antioxidant/oxidant status in the tissue and serum samples, catalase (CAT), superoxide dismutase (SOD), total glutathione (GSH), nitric oxide (NO), malonyl dialdehyde (MDA) and total anti-oxidant capacity (T-AOC) were measured after 7 and 28 days. In the early phase, serum T-AOC levels were significantly elevated in the EG+P100 ($p=0.0062$) compared to the control, while in the late phase, it was elevated in the EG+P50 ($p=0.037$) and EG+V ($p=0.009$) compared to the EG only group. Propolis administration was observed to dramatically decrease crystal deposition ($p<0.0001$) and was more effective in the prevention of oxalate-induced renal injury than verapamil. Propolis being a natural product with almost none adverse effects elevates its value as a future approach to urolithiasis.

Keywords: Hyperoxaluria; propolis; verapamil; antioxidant; crystal deposition.

1. Introduction

Propolis, a honey-bee product, is known to comprise the most powerful antioxidant agents. Over hundreds of compounds have been identified related to propolis. The main groups can be summarized

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as phenolic acids, flavonoids, terpenes, lipid-wax substances, beeswax, bio-elements, essential oils and sugars [1-3]. Phenolic compounds, namely phenolic acids, phenolic aldehydes, phenols and their esters, ketophenols, coumarins, eugenol, anethole, hydroquinone, pterostilbene, naphthalene, etc., determines the quality and type of propolis [1] and especially flavonoids (caffeic acid phenethyl ester (CAPE), cinnamic acid etc.) are responsible for its biological activity [4,5]. The compounds that constitute propolis have attracted attention and recently studies have been conducted concerning its vasoprotective, anti-oxidant, anti-inflammatory, anti-cancer and anti-angiogenic features [6,7].

Although hyperoxaluria is common in the general population, there are no specific treatment approaches. Renal tubular epithelium is one of the major sites of oxalate- induced injury where sustained hyperoxaluria and subsequent calcium oxalate (CaOx) crystal formation/deposition could be damaging to the renal tubular cells [8-11]. In the case of oxalate formation, increased reactive oxygen species (ROS) directly damage the renal cells, furthermore; obstruction of the urinary channels and the subsequent elevation of intrarenal tubular pressure is an indirect effect. This entity is recognized as crystal induced acute kidney injury. ROS effect tissues by impairing different pathways related to DNA damage and protein modifications. Moreover, increased ROS in some systematic diseases may also lead to kidney damage. The CaOx crystals and/or oxalate ions play a vital role in the formation of urinary calculi [12-14]. In order to understand the mechanism of cell damage during hyperoxaluria, stimulated lipid peroxidation in tubular cells, ROS and its formation causing oxidative stress should be evaluated all together. Hyperoxaluria-induced tubular ischemia may have a major role in initiating the programmed sequence of events leading to cell death [15,16].

In this study, it was aimed to investigate the presence and extent of intratubular crystal formation along with the oxidant/antioxidant status in the kidney tissues and serum samples, and the conceivable protective effects of two agents, verapamil and propolis, on these changes induced by hyperoxaluria in a rat model. Moreover, the focus of this paper was to compile the setting inducing oxidative stress (OS) in kidneys and to perform a comparative analysis between verapamil and propolis in terms of their potential preventive affects on a urolithiasis model in rats.

2. Materials and Methods

2.1. Animals

A total of 76 female Sprague–Dawley rats (350-400 g each) were included to this study with the approval of the Ethical Committee of the Pendik Institute of Veterinary Control and Research (94-14/013). All animals were kept in standard room conditions (23±1 ^{0}C and 55%±5 humidity) with day and night periods of 12 hours. Animals were then divided into four groups of treated animals and the untreated control group (Group 1) as early (7 days) and late (28 days) phase follow-up groups. In Group 2 (Ethylene Glycol (EG) only), hyperoxaluria was induced by administering animals 0.75% EG containing drinking water [17]. In addition to hyperoxaluria induction, animals in Group 3 were given propolis (50 mg/kg through feeding tube); Group 4 were given high dose propolis (100 mg/kg through feeding tube); and Group 5 were given verapamil (1 mg/kg, through feeding tube). All groups were evaluated in early (7 days) and late (28 days) phases (Table 1). Their daily water consumptions were similar.

Table 1. Study groups

Time	Group 1 Control (n)	Group 2 EG (n)	Group 3 EG+Propolis 50 (n)	Group 4 EG+Propolis 100 (n)	Group 5 EG+Verapamil (n)
Early Phase (7 Days)	6	8	8	8	8
Late Phase (28 Days)	6	8	8	8	8

Following euthanasia via cervical dislocation, bilateral flank incisions were performed on the animals to remove both their kidneys for the histopathologic evaluation of crystal formation (under light microscopy) together with oxidant/antioxidant status in the tissue and serum samples by using the ELISA kits for catalase (CAT), superoxide dismutase (SOD), total glutathione (GSH), nitric oxide (NO), malonyl dialdehyde (MDA), total anti-oxidant capacity (T-AOC).

2.2. Experimental Design

Kidney tissue samples were collected following the cervical dislocation of the rats. Tissues were immersed in liquid nitrogen and pounded using a ceramic mortar and pestle. Then, they were homogenized using TissueRuptor® (Qiagen, Netherlands) and equilibrated to 1mg tissue/1 ml PBS (Phosphate Buffered Saline, Biochrome). Blood samples were acquired before euthanization and serum samples were separated for the ELISA tests.

2.3. ELISA Tests

The ELISA tests (YH-Biosearch, China) of catalase (CAT), superoxide dismutase (SOD), total glutathione (GSH), nitric oxide (NO), malonyl dialdehyde (MDA), and total anti-oxidant capacity (T-AOC) were used to assess the oxidant/antioxidant status of the tissue and serum samples.

2.4. Histological Analysis

Evaluations of renal crystal deposition and calcification in tubules were performed under light microscopy for the late phase (28^{th} day) in order to appraise the utmost renal stone formation. Tissues were embedded in paraffin and stained with hematoxylin and eosin (HE) for histopathological evaluation. Detection of crystallization in the frozen sections of the kidneys of the rats treated only with EG or EG and propolis (EG+PRO) or EG and verapamil (EG+V), were qualitatively assessed by histopathological evaluation.

For each study group, crystal formations were calculated in a total of 240 renal tubules, forty tubules from 6 different microscopic fields.

2.5. Preparation and Characterization of the Ethanolic Extract of Propolis

Propolis was obtained from Altiparmak Inc. (Istanbul, Turkey). According to the validity and reliability tests performed by Altiparmak Inc. in Apilab Laboratories, the propolis sample was stated as suitable for consumption and no biological or heavy metal contamination were detected. It was dissolved in 70% (v/v) ethanol and the final concentration was set as 30mg propolis/1g solution (w/w). The ethanolic extract was incubated overnight at 37 C° with occasional shaking and then filtered through a syringe filter (0.22 micron).

2.6. Total Phenolic Contents and Total Antioxidant Activity Analysis (mg GAE/g)

The total phenolic content (TPC) in propolis was determined using Folin–Ciocalteu reagent. The findings were demonstrated as mg gallic acid equivalent (GAE) 100 g^{-1} sample [18]. Total antioxidant capacity (TAC) was appraised using the 2,2-azinobis(3-ethylbenzothiazoline)-6-sulfonic acid (ABTS), 1,1-diphenyl-2-picrylhydrazyl (DPPH), ferric reducing antioxidant power (FRAP) and cupric ion reducing antioxidant capacity (CUPRAC) assays [19-22]. In all assays, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) was utilized as a standard and the findings were demonstrated as mg Trolox equivalent (TE) 100 g^{-1} sample.

Compounds and standards were purchased from Sigma-Aldrich, Merck, ExtraSynthese, Genay-France. LC-MS/MS analyses were performed using a Zivak® HPLC and Zivak® Tandem Gold Triple Quadrupole (Istanbul, Turkey) mass spectrometer, equipped with a Synergy Max C18 column (250 x 2 mm i.d., 5µm particle size) according to a previous study in Organic Chemistry

Laboratory, Chemistry Group, National Metrology Institute, TUBITAK, Kocaeli, Türkiye [23] (Table 2, Table 3 and supporting information).

Table 2. Phenolic contents of propolis sample by LC-MS/MS

Phenolic Content	(X±SD)
Kaempferol	1135.2±125.12
Fumaric acid	4975.50±559.17
Pyrogallol	5625.09±691.86
p-OH benzoic acid	478.47±41.63
p-Coumaric acid	4227.78±753.45
Caffeic acid	7978.20±1598.88
t-Ferulic acid	3121.84±289.92
Quercetin	734.88±124.67
Ellagic acid	52.08±4.62
Isorhamnetin	571.95±76.46
Quercetagetin-3,6-dimethyl ether	1819.86±378.14
Chlorogenic acid	301.93±42.62
Rosmarinic acid	139.36±14.10
Rutin	1136.92±89.66
Gallic acid	249.25±18.96
Salvigenin	2597.53±227.36
Penduletin	698.78±80.43
Total Phenolic Substance (mg GAE/g)	143± 15

X: Amount of substances as mg/kg
SD: Values are given as Mean ± SD

Table 3. Total antioxidant capacity of studied propolis sample

Applied Method	(mg TEAC/g)*
ABTS	207 ± 36
CUPRAC	575 ± 31
DPPH	151 ± 33
FRAP	140± 18

*Values are given as Mean ± SD

2.7. Statistical Analysis

The statistical analysis was performed using the GraphPad Prism 6.0 software. The data were analyzed by 2-way ANOVA test with Bonferroni method correction (alpha: 0.01) to find differences between the control and treated groups.

3. Results and Discussion

In this study, in the early period, serum MDA levels, a lipid peroxidation product, were similar among all groups compared to the control, while in the late period, a significant decrease was observed only in the EG+V group (Figure 1, Table 4). Conversely, several studies conducted on the possible preventive effects of various agents on urolithiasis showed higher tissue MDA levels following EG administration in rats [24,25] and propolis has been demonstrated to decrease lipid peroxidation (MDA levels), as part of its antioxidant activity [26]. In this study, a slight decrease was observed in the EG+P100 group. This may be due to that the MDA test is not sufficient to determine oxidative stress. In fact, currently researches are debating over the reliability and efficiency of MDA testing in determining oxidative stress [27].

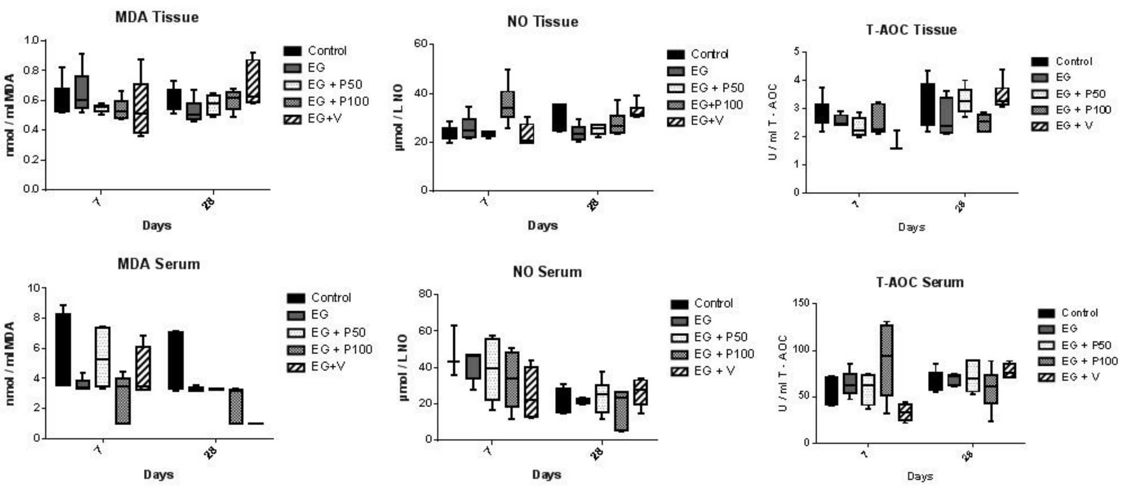

Figure 1. Serum and tissue levels of MDA, NO and T-AOC

In the early phase, serum NO levels were decreased in the EG+V group compared to both control and EG only groups. In the tissue samples, NO levels were elevated with propolis administration, yet reached significance in the EG+P100 group compared to the control group (p=0.0001, Figure 1, Table 5). Moreover, in the tissue samples compared to the EG only group, NO elevation was observed in EG+ P100 and decrease in the EG+V groups. In the late phase, NO levels seem unvaried aside from the elevation observed in the EG+V group when compared to the EG only group, indicating that Verapamil shows its efficiency in the long term. Since NO is a molecule metabolized shortly after its formation; our current findings indicate that the antioxidant systems eliminate it from the serum and tissue effectively until late phase as expected. One of the numerous benefits of propolis is its immune regulatory impact. Propolis has been shown to elevate NO levels [26], which is consistent with our findings.

Table 4. Estimation of the serum and tissue levels of MDA within the study groups

Days	**MDA**	**SERUM**		**TISSUE**	
		Mean ± SD	**P Value**	**Mean ± SD**	**P Value**
7	Control	3.69 ± 1.17		0.62 ± 0.11	
	Control vs. EG	4.02 ± 0.44	0.758	0.65 ± 0.16	> 0.9999
	Control vs. EG+P50	5.38 ± 0.84	0.262	0.55 ± 0.03	> 0.9999
	Control vs. EG + P100	2.68 ± 0.71	0.464	0.54 ± 0.07	0.9896
	Control vs. EG + V	4.26 ± 0.88	0.711	0.54 ± 0.18	0.8196
	EG vs. EG+P50	5.383 ± 2.06	0.025	0.55± 0.03	0.3425
	EG vs. EG + P100	2.814 ± 1.46	0.679	0.54± 0.07	0.2667
	EG vs. EG + V	4.046 ± 1.40	> 0.9999	0.54± 0.18	0.213
28	Control	3.28 ± 0.77		0.61 ± 0.08	
	Control vs. EG	3.34 ± 0.04	0.585	0.53 ± 0.08	0.8454
	Control vs. EG+P50	3.02 ± 0.29	0.671	0.57 ± 0.06	> 0.9999
	Control vs. EG + P100	3.71 ± 0.96	0.828	0.60 ± 0.07	> 0.9999
	Control vs. EG + V	0.99 ± 0.01	<0.0001 ****	0.70 ± 0.15	0.581
	EG vs. EG+P50	3.28 ± 0.03	> 0.9999	0.57±0.06	> 0.9999
	EG vs. EG + P100	2.49 ± 1.16	0.6067	0.60 ± 0.07	0.7826
	EG vs. EG + V	0.98 ± 1.16	0.0023 **	0.70 ± 0.15	0.0323

Table 5. Estimation of serum and tissue levels of NO within the study groups

Days	NO	SERUM		TISSUE	
		Mean ± SD	**P Value**	**Mean ± SD**	**P Value**
7	Control	45.49 ± 9.11		23.85 ± 3.08	
	Control vs. EG	42.56 ± 7.68	> 0.9999	26.05 ± 4.99	> 0.9999
	Control vs. EG+P50	39.2 ± 15.29	> 0.9999	23.33 ± 1.07	> 0.9999
	Control vs. EG + P100	33.14 ± 15.29	0.1514	35.46 ± 8.10	0.0001 ***
	Control vs. EG + V	26.3 ± 11.33	0.0068 **	22.63 ± 4.17	> 0.9999
	EG vs. EG+P50	39.2 ± 15.29	> 0.9999	23.33 ± 1.07	0.8944
	EG vs. EG + P100	33.14 ± 15.29	0.3915	35.46 ± 8.09	0.0023 **
	EG vs. EG + V	26.3 ± 11.33	0.0331**	22.63 ± 4.16	0.5773
28	Control	21.56 ± 5.36		29.65 ± 4.98	
	Control vs. EG	21.67 ± 1.55	> 0.9999	23.96 ± 3.23	0.1191
	Control vs. EG+P50	24.20 ± 9.09	> 0.9999	25.29 ± 2.00	0.3709
	Control vs. EG + P100	18.41 ± 10.35	> 0.9999	27.69 ± 5.01	> 0.9999
	Control vs. EG + V	26.97 ± 6.85	> 0.9999	32.55 ± 3.39	> 0.9999
	EG vs. EG+P50	24.2 ± 9.09	> 0.9999	25.29 ± 2.00	> 0.9999
	EG vs. EG + P100	18.41 ± 10.34	> 0.9999	27.69 ± 5.01	0.4684
	EG vs. EG + V	26.97 ± 6.84	> 0.9999	32.55 ± 3.38	0.0057

In the early phase, serum and tissue T-AOC levels were elevated in the EG+P100 group, yet decreased in the EG+V group, indicating that propolis is enhancing TAOC in the early period. In the late phase, when compared to EG only group EG+P50 and EG+V groups also supported T-AOC (p= 0.0062, Table 6). The fact that T-AOC displays an additive impact of all antioxidant systems in the tissue, it may not be as specific as its constituents.

Table 6. Estimation of the serum and tissue levels of T-AOC within the study groups

Days	T-AOC	SERUM		TISSUE	
		Mean ± SD	**P Value**	**Mean ± SD**	**P Value**
7	Control	54.19 ± 13.80		2.82 ± 0.52	
	Control vs. EG	63.74 ± 13.16	> 0.9999	2.57 ± 0.19	> 0.9999
	Control vs. EG+P50	63.66 ± 15.31	> 0.9999	2.32 ± 0.33	0.3302
	Control vs. EG + P100	89.00 ± 40.54	0.0062 **	2.54 ± 0.50	> 0.9999
	Control vs. EG + V	33.25 ± 7.19	0.1968	1.90 ± 0.35	0.0074 **
	EG vs. EG+P50	63.66 ±15.31	> 0.9999	2.32 ± 0.33	0.7023
	EG vs. EG + P100	89 ± 40.54	0.082	2.53 ± 0.50	0.9992
	EG vs. EG + V	33.25 ± 7.19	0.0257	1.89 ± 0.35	0.0297
28	Control	66.83 ± 11.40		3.13 ± 0.80	
	Control vs. EG	68.90 ± 5.73	> 0.9999	2.65 ± 0.64	0.3857
	Control vs. EG+P50	71.00 ± 16.15	> 0.9999	3.30 ± 0.47	> 0.9999
	Control vs. EG + P100	58.42 ± 21.44	> 0.9999	2.51 ± 0.28	0.1362
	Control vs. EG + V	77.67 ± 7.91	> 0.9999	3.44 ± 0.49	> 0.9999
	EG vs. EG+P50	71 ±16.14	> 0.9999	3.298 ± 0.47	0.0376
	EG vs. EG + P100	58.42 ± 21.43	> 0.9999	2.512 ± 0.28	0.9285
	EG vs. EG + V	77.67 ± 7.90	> 0.9999	3.435 ± 0.49	0.009**

Furthermore, propolis also has been shown to increase SOD and GPx owing to its antioxidant properties [26]. In this study, in the early period, only difference was observed in the EG+P50 group in terms of serum SOD levels compared to the control. In the late phase, compared to the EG only group, serum SOD levels were elevated, while tissue SOD levels were decreased in the EG+P50 and EG+V groups (Table 7, Figure 2). However, antioxidant administration such as green tea has been shown to elevate SOD levels in a similar model [28]. Serum GSH levels were decreased both in early (p= 0.005-0.001) and late phases (p<0.0001) in all groups (Table 8). In the early phase there was no difference observed in terms of serum or tissue CAT levels, while in the EG+V, EG+ P50 and EG+ P100 groups tissue CAT levels were decreased (p=0.001 Figure 2, Table 9).

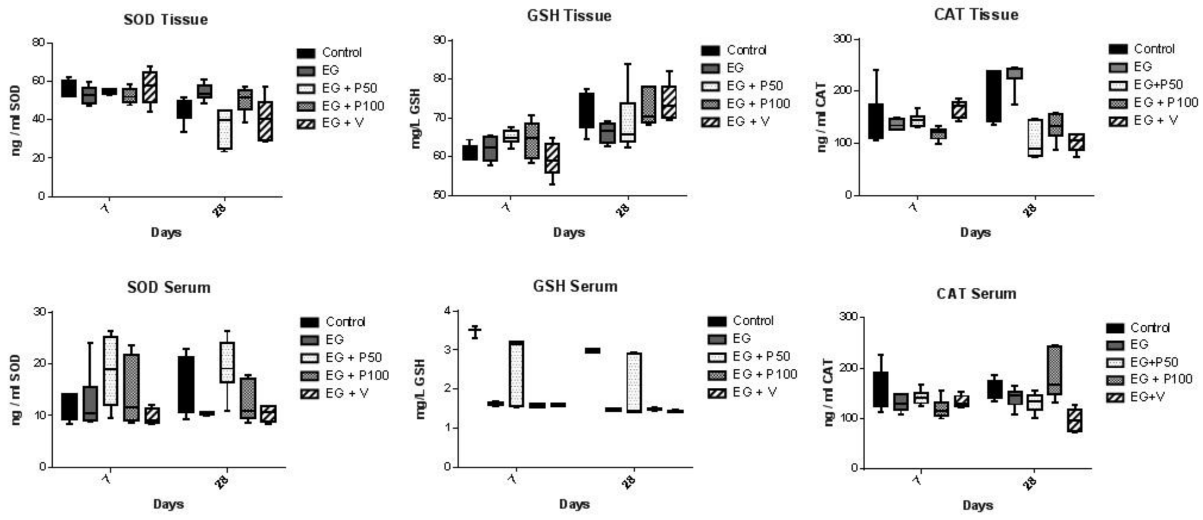

Figure 2. Serum and Tissue SOD, GSH and CAT levels.

Table 7. Estimation of the serum and tissue levels of SOD within study groups

Days	SOD	SERUM		TISSUE	
		Mean ± SD	**P Value**	**Mean ± SD**	**P Value**
7	Control	11.41 ± 2.26		54.95 ± 4.30	
	Control vs. EG	12.58 ± 5.82	> 0.9999	52.97 ± 4.84	> 0.9999
	Control vs. EG+P50	18.55 ± 7.51	0.0406	54.59 ± 1.34	> 0.9999
	Control vs. EG + P100	14.30 ± 6.43	> 0.9999	52.54 ± 3.64	> 0.9999
	Control vs. EG + V	9.76 ± 1.43	> 0.9999	57.31 ± 7.94	> 0.9999
	EG vs. EG+P50	18.55 ± 7.51	0.1001	54.59 ± 1.34	> 0.9999
	EG vs. EG + P100	14.3 ± 6.43	> 0.9999	52.54 ± 3.64	> 0.9999
	EG vs. EG + V	9.76 ± 1.42	0.9128	57.31 ± 7.94	0.7996
28	Control	15.48 ± 5.74		45.15 ± 6.40	
	Control vs. EG	10.48 ± 0.26	0.2667	54.41 ± 4.22	0.0645
	Control vs. EG+P50	19.53 ± 5.21	0.5462	36.69 ± 9.27	0.1082
	Control vs. EG + P100	12.56 ± 3.65	> 0.9999	50.26 ± 6.62	0.7036
	Control vs. EG + V	10.40 ± 1.39	0.2506	39.77 ± 10.28	0.6145
	EG vs. EG+P50	19.53 ± 5.21	0.0055**	36.69 ± 9.27	0.0001***
	EG vs. EG + P100	12.56 ± 3.65	> 0.9999	50.26 ± 6.62	0.8596
	EG vs. EG + V	10.4 ± 1.39	> 0.9999	39.77 ± 10.28	0.0014**

Therefore, we suggest that tissue CAT may be used to eliminate oxidant effect delivered via EG administration in the late period (p<0.0001). However, in the late phase, when compared to the EG only group, this decrease seems to fade, while in the EG+V group a significant elevation was observed. Thus, propolis was effective starting from the early phase but verapamil was effective in later periods (Figure 2).

Table 8. Estimation of serum and tissue levels of GSH within all groups

Days	GSH	SERUM		TISSUE	
		Mean ± SD	**P Value**	**Mean ± SD**	**P Value**
7	Control	3.50 ± 0.11		61.12 ± 1.92	
	Control vs. EG	1.62 ± 0.04	< 0.0001 ****	62.09 ± 3.31	> 0.9999
	Control vs. EG+P50	2.64 ± 0.83	0.0005 ***	65.05 ± 1.83	0.5106
	Control vs. EG + P100	1.57 ± 0.04	< 0.0001 ****	64.52 ± 4.71	0.7479
	Control vs. EG + V	1.60 ± 0.02	< 0.0001 ****	59.38 ± 3.88	> 0.9999
	EG vs. EG+P50	2.639 ± 0.83	0.0002***	65.05 ± 1.83	0.7952
	EG vs. EG + P100	1.571 ± 0.04	> 0.9999	64.52 ± 4.71	> 0.9999
	EG vs. EG + V	1.6 ± 0.02	> 0.9999	59.38 ± 3.88	0.9278
28	Control	2.97 ± 0.04		71.52 ± 4.97	
	Control vs. EG	1.47 ± 0.02	< 0.0001 ****	66.25 ± 2.55	0.1739
	Control vs. EG+P50	1.93 ± 0.77	< 0.0001 ****	68.75 ± 7.89	> 0.9999
	Control vs. EG + P100	1.49 ± 0.03	< 0.0001 ****	72.34 ± 4.51	> 0.9999
	Control vs. EG + V	1.43 ± 0.02	< 0.0001 ****	74.20 ± 4.98	> 0.9999
	EG vs. EG+P50	1.928 ± 0.76	0.1657	68.75 ± 7.89	> 0.9999
	EG vs. EG + P100	1.492 ± 0.02	> 0.9999	72.34 ±4.51	0.0769
	EG vs. EG + V	1.433 ± 0.02	> 0.9999	74.2 ± 4.98	0.0129

In our previous study [29], the efficiency of verapamil was shown on an urilithiasis rat model. In this study, we further evaluated the capability of propolis in comparison with Verapamil in a similar rat model. Considering these findings and previous data, both hyperoxaluria and CaOx crystal formation are certainly damaging to renal epithelial cells due to oxidative stress. Scavenging enzyme alterations adjust the T-AOC of the kidneys and crystal deposition signifying the high possibility of such specific changes due to the hyperoxaluria induced ischemic injury in renal tubular cells. Moreover, studies indicated that cellular injury noted in the renal papillary tubular epithelial cells due to hyperoxaluria-induced ischemia, might induce cell degradation, which could be at the bottom of the pathologic progression of urolithiasis. These findings confirm that during the hyperoxaluric phase some apoptotic events do take place in response to oxidative stress. Very limited crystallization was observed during the early phase in the affected kidneys as an incipient of crystal formation following hyperoxaluria. Furthermore, the verapamil and propolis applications were able to limit crystallization during late phase. Animals receiving Verapamil treatment demonstrated mildly limited oxalate crystal formation when compared with the EG only group, similar with the control group. During late phase, however, propolis was able to limit crystal formation significantly. Hereby, according to the microscopic evaluations while verapamil was capable of restricting crystal formation during late phase, propolis treatment was able to exhibit a better impact (Table 10, Figure 3). Furthermore, microscopically a far much lower stone formation and macroscopically softer and brighter kidneys with a color close to normal appearance were observed in the animals administered with propolis (data not shown).

Integrative and preventive therapies can possibly provide additional benefits to the modern treatment methods against urolithiasis. It is well known that developing an effective prophylactic approach to urolithiasis will promote the inhibition of new crystal formation. Many current models of calcium oxalate (CaOx) stone disease suggest that the generation of ROS and subsequent lipid peroxidation is included in the tubular cell damage and apoptotic mechanism for stone formation. Typically, cells have various antioxidant systems to limit this molecular process, including enzymatic (SOD, CAT and glutathione peroxidase-GPx) and non-enzymatic (vitamins E, A and C) approaches. Thus, the oxidant/antioxidant balance is crucial for cell sensitivity against free-radical damage and an increased production of ROS in response to hyperoxaluria activates adaptive mechanisms in the kidney by up-regulating the antioxidant defense systems such as SOD, CAT and GSH. Moreover, recurrent idiopathic CaOx stone formers, with stones or simply crystals, were shown to concurrently have antioxidant deficiency [30].

Table 9. Estimation of serum and tissue levels of CAT within all groups

Days	CAT	SERUM		TISSUE	
		Mean ± SD	**P Value**	**Mean ± SD**	**P Value**
7	Control	150.09 ± 39.98		146.7 ± 48.71	
	Control vs. EG	131.30 ± 15.18	0.8114	135.6 ± 10.12	> 0.9999
	Control vs. EG+P50	142.30 ± 14.73	> 0.9999	144.6 ± 12.91	> 0.9999
	Control vs. EG + P100	119.80 ± 19.70	0.1728	118.8 ± 11.58	0.4037
	Control vs. EG + V	134.10 ± 11.78	> 0.9999	163.8 ± 16.28	> 0.9999
	EG vs. EG+P50	142.3 ± 14.73	> 0.9999	144.6 ± 12.91	> 0.9999
	EG vs. EG + P100	119.8 ±19.70	> 0.9999	118.8 ± 11.57	0.5282
	EG vs. EG + V	134.1 ± 11.78	> 0.9999	163.8 ± 16.27	0.077
28	Control	158.47 ± 20.03		176.04 ± 48.98	
	Control vs. EG	142.00 ± 18.94	> 0.9999	231.40 ± 28.14	0.0066 **
	Control vs. EG+P50	132.40 ± 18.38	0.3218	103.40 ± 33.70	0.0003 ***
	Control vs. EG + P100	184.30 ± 47.57	0.33	132.90 ± 25.70	0.0501
	Control vs. EG + V	97.34 ± 21.31	0.0005 ***	101.80 ± 17.21	0.0002 ***
	EG vs. EG+P50	132.4 ±18.38	> 0.9999	103.4 ± 33.70	**< 0.0001***
	EG vs. EG + P100	184.3 ±47.57	0.0097**	132.9 ± 25.70	**< 0.0001***
	EG vs. EG + V	97.34 ± 21.30	0.0061**	101.8 ± 17.21	**< 0.0001***

Many oxidative stress markers are increased in the experimental rat kidney models. For instance, MDA, a lipid peroxidation marker, is a commonly used oxidative stress marker although it can be found in fluctuating levels owing to dietary habits and lifestyle. Urinary 8-hydroxydeoxy guanosine (8-OHdG) levels, an indicator of oxidative damage on DNA, was elevated in patients with kidney stones and was related to tubular damage [31]. Studies conducted on tissue cultures displayed the influence of free radicals on intense inflammation and reproduction of numerous crystallization modulators. Renal cells secrete superoxide radicals in response to CaOx crystals, and antioxidants and free radical scavengers may conceivably eliminate the consequent cell damage. It has been shown that CAT and SOD, as free radical scavengers, prevented the damage induced by oxalate *in vitro* in animal kidney epithelial cell lines [32]. Furthermore, supplementary to the evaluation of the scavenging enzymes, total antioxidant capacity (T-AOC) is now commonly used in order to appraise the oxidant/antioxidant status. It was also considered as a favorable route to estimate the extent of oxidative stress [33,34].

Elevated levels of oxidative stress have been reported in pathological states of the kidneys by numerous researchers [35]. Huang et al. demonstrated increased CAT and manganese superoxide dismutase (MnSOD) enzyme activities in rats with EG-induced early stage urolithiasis. All antioxidant enzyme activities were decreased aside from CAT on day 42 [17, 36]. Increased ROS *in vitro*, and NO synthase (iNOS) and NFkB expressions *in vivo* indicate ascended oxidative stress in rat kidneys [32,37]. Furthermore, it has been shown that the Extracorporeal Shock Wave Lithotripsy (ESWL) treatment induces oxidative stress and reduces the antioxidant and trace element levels in rat kidneys [38]. In addition, renal tubular apoptosis have been associated with incremented ROS in rats [39]. Moreover, elevated renal ROS levels were also reported in obstructed kidneys *in vivo* along with the impairment of the prominent antioxidant enzymes, SOD, CAT and glutathione peroxidase (GPx) [40]. Various studies have been conducted on the favorable effect of the antioxidant and reactive oxygen scavenger agents against urinary obstruction [41], infection [42], and ischemia-reperfusion injury [43] induced renal damages. Following the observation of hyperoxaluric crystal deposition in the renal parenchyma and cellular injury in the tubular epithelium, preventive effect of the protective agents was investigated. Administration of antioxidants in hyperoxaluric rat models reduced renal injury, thus lipid peroxide production and CaOx crystal deposition in the kidneys indicated the involvement of ROS in hyperoxaluria-induced renal injury [44]. Calcium channel-blocking agents (CCB), anti-inflammatory agents (as Tutukon®) or vitamin E used as free radical scavenger agents were observed to restrict histologic changes and crystal deposition by minimizing free oxygen radical-induced alterations in parenchymatous organs [29,45-51]. In order to increase the blood flow to the affected organ, including kidneys, and regain the normal physiology, CCB agents are used to induce vasodilation and reduction of peripheral resistance, which is shown to successfully confine the ischemia-related alterations. Moreover, lemon juice has been proposed in the treatment of kidney stones due to its citrate and antioxidant content [52,53]. Citrate prevents crystal formation by inhibiting the saturation of calcium and the antioxidant content limits the renal tubular damage. Therefore, we suggest that elevated renal oxidative stress and the subsequent functional impairment of the endothelial cells may be the underlying inducer and/or initiator of urolithiasis development.

Among many fascinating features of propolis, there are protective effects such as anti-inflammatory [54,55], xanthine oxidase (XOD) inhibitory and hypouricemic action [56-58], renoprotective, diuretic, antimicrobial, antioxidant and immunomodulator effects. Xanthine oxidase is an enzyme that generates reactive oxygen species and is further metabolized to uric acid [45]. A xanthine oxidase inhibitor, namely allopurinol, is known to inhibit the ischemia and reperfusion damage in many organs including kidneys and is also a component of the organ storage solution used for transplantations [59]. Flavonoids in propolis are extremely powerful antioxidants [60]. CAPE, an active compound of propolis, was investigated for lithium induced renal toxicity on rats and CAPE treatment was suggested to be protective [61] In addition, Holoch and Tracy observed a link between low serum antioxidant levels and incidences of kidney stones noted by the patients. There are studies that demonstrated the elimination of oxidative stress in kidneys to various degrees by propolis [62, 63]. Moreover, some flavonoids are effective in ameliorating blood pressure via escalating water and electrolyte excretion (as Na^{++} reabsorption) from kidneys [64-67]. These mechanisms may have significance in stone formation.

Consequently, both visible crystal deposition and tubular injury due to oxidative stress might be confined by blood flow regulators (calcium antagonists) and antioxidant agents (propolis). Although verapamil is an efficient agent to prevent oxalate- induced AKI urolithiasis, it is a Ca channel blocker, thus it cannot be used for long durations since it will slow the heart rate and decrease the blood pressure of the patients. However, propolis treatment is non-toxic in appropriate usage and shown to be renal-protective with its high phenolic content and many properties beyond antioxidant. These findings suggest that propolis may be used to prevent stone formation in people prone to urolithiasis or for accidental EG induced renal injury.

Table 10. Comparative effects of ethylene glycol, verapamil and propolis on renal oxalate crystal deposition

	Administrated Agents		
	Ethylene glycol (n=6)	**Verapamil (n=6)**	**Propolis (n=6)**
Oxalate crystal deposition (X±SEM)	13.67±0.88 *	9.00± 1.09 **	2.67±0.49 ***

*, p value for EG vs. Verapamil = 0.0078 (95% Confidence interval= (-)7.800- (-)1.533)
**, p value for Verapamil vs. Propolis = 0.0004 (95% Confidence interval= (-)9.011- (-)3.655)
***, p value for Propolis vs. Ethylene glycol = 0.0001 (95% Confidence interval= (-)13.25- (-)8.747)

A

B

C

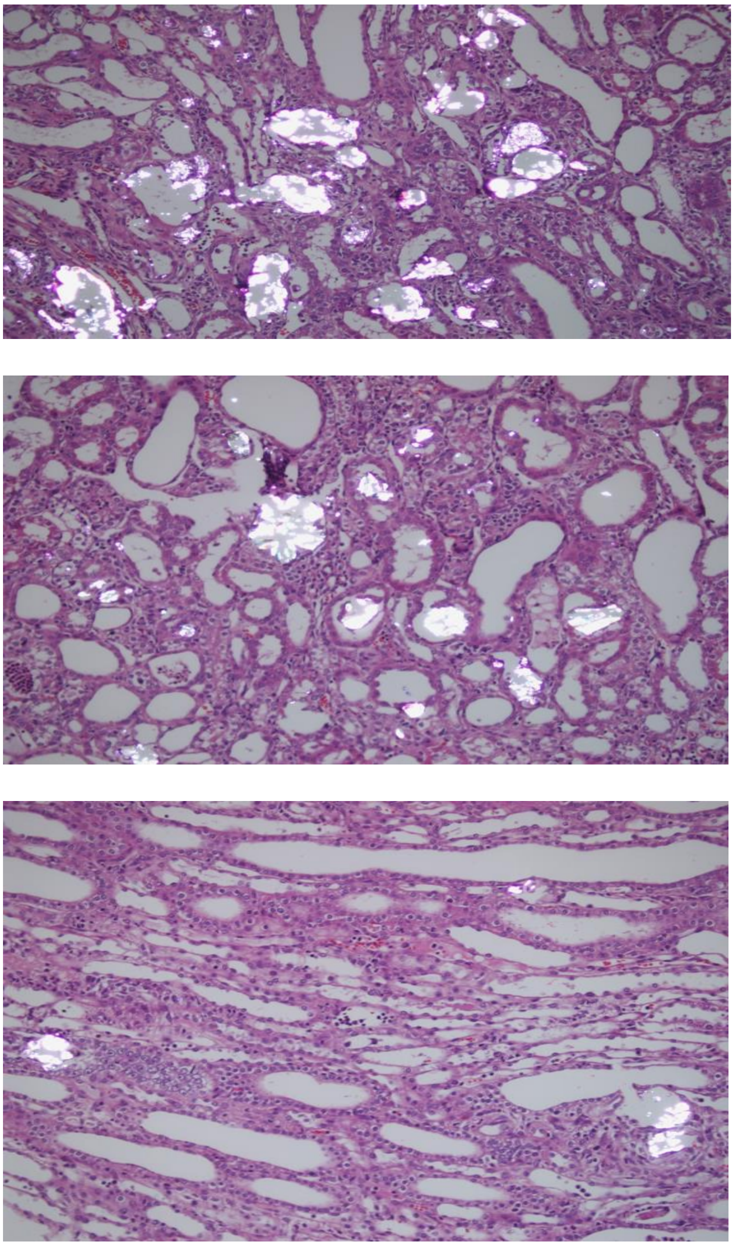

Figure 3. Evident crystal formation (EG group, dense deposition) in an animal undergoing hyperoxaluria induction (H&E, Olympus polarizer filter, original magnification X200).
A: without any protective agent at late phase
B: with Verapamil application at late phase
C: with Propolis 50 mg/kg application at late phase

No metabolic evaluation was performed on rat urine, since the cages that were used were not suitable for urine sample collection in this study. Additionally, no assessments were made for quantitative histological analysis on the renal tissue samples. These aspects may be further evaluated in order to investigate the renal-related metabolic effects of propolis.

4. Conclusions

The presented findings deliver novel and substantial evidence on crystal-induced oxidative damage to the renal tissue, which provides a favorable environment for individual CaOx crystal attachment and subsequent development of kidney stones *in vivo*. Propolis treatment confined the CaOx crystal deposition in the kidney more than calcium channel blockers, via preventing the antioxidant imbalance in the tissues caused by hyperoxaluria. Moreover, many researchers now recognize a wide range of favorable impact of propolis. In this model, the predominant effect of propolis may be not only its antioxidant effect but also to anti-inflammatory, immunomodulatory and anti-ischemic activities owing to its high phenolic content (Table 2, supporting information S1). Therefore, propolis could be included to the prevention of hyperoxaluria-induced kidney stone formation. In this context, further studies should be carried out on their bioavailability, most appropriate preparations, and designating the safe and favorable doses to prevent or treat urolithiasis.

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Informed consent

Informed consent was not required (Experimental animal study). The authors have declared that no conflict of interest exists.

Ethical approval: Study approval was obtained from the Ethical committee of the Institute of Veterinary Control and Research, Pendik-İstanbul (94-14/013).

Supporting Information

Supporting information accompanies this paper on http://www.acgpubs.org/RNP

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