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Original Article BIOCHEMISTRY

The effect of *Myrtus communis* L. extract on nephrolithiasis model in rats

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ABSTRACT

OBJECTIVE: Nephrolithiasis is a common urological disease that can lead to renal failure. Oxidative stress has been shown to be a contributing factor for nephrolithiasis and many agents have been studied to prevent and treat oxidative stress-related nephrolithiasis and renal damage. *Myrtus communis* (MC) extract has been shown to be an important antioxidant in different animal models. In this study, MC extract was administered preventively or therapeutically to rats with kidney stones, and its effectiveness was investigated.

METHODS: Wistar albino rats were divided into four groups (n=8); control (C), ethylene glycol (EG), EG+preventive MC, and EG+curative MC groups. The nephrolithiasis model was created by adding 0.75% EG to drinking water for 8 weeks. Ultimately, 24-hour urine was collected to measure calcium, citrate, and creatinine levels. After decapitation, kidney tissues were harvested for histological analyses, measurement of osteopontin and 8-hydroxydeoxyguanosine (8-OHdG) levels, and N-acetyl-β-glucosaminidase (NAG), myeloperoxidase (MPO) and caspase-3 activities.

RESULTS: In 24-hour urine samples, calcium, citrate and creatinine levels were decreased in the EG group, while oxalate levels were increased and in treatment groups these parameters returned to control levels. MPO, 8-OHdG, caspase-3 and NAG activity were significantly increased in tissue and these changes were reversed in both MC groups. Histological findings also supported the biochemical parameters.

CONCLUSION: MC can reduce oxidative stress and histopathological changes in kidney tissues in rat nephrolithiasis model when used as either a preventive or therapeutic agent. If supported with further clinical trials, MC might have clinical implications in preventing oxidative renal cell injury and ultimately kidney stone formation.

Keywords: Ethylene glycol; hyperoxaluria; nephrolithiasis; oxidative stress; renal insufficiency.

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Nephrolithiasis is an important chronic urological condition that affects around 10% of the population [1]. Due to its frequency and its recurrent nature, nephrolithiasis is one of the major etiological factors of renal insufficiency ultimately creating a great financial health burden [2]. Thus, studies on the prevention of stone formation and as well as therapeutic measures are gaining more popularity each day [3].

Stone formation is a series of processes involving nucleation, crystal growth, aggregation and crystal retention [3, 4]. There are promoters and inhibitors of stone formation. The inhibitors act on any of the aforementioned stages to reduce stone formation. Some of the known inhibitors are alkaline pH, citrate, pyrophosphate, magnesium and glycoproteins such as osteopontin, Tamm-Horsfall protein, urinary prothrombin fragment 1, glycosaminoglycans, nephrocalcin [5].

On the other hand, oxidative stress has been shown to be a major contributor to both the initiation of the stone formation process as well as the ongoing stone growth [6, 7]. It has been previously shown that crystal deposition is associated with reactive oxygen species and increased expression of molecules that have key roles in inflammatory cascades in animal models [7].

One of the most frequently used nephrolithiasis models in rats is the ethylene glycol-induced nephrolithiasis model [8, 9]. Ethylene glycol (EG) is a colourless, odourless soluble chemical that is commonly used as an industrial solvent. After hepatic metabolisation with alcohol dehydrogenase, EG is converted into toxic organic acids such as glycolaldehyde, glycolic acid, glyoxylic acid and oxalic acid. Oxalic acid reacts with calcium to create calcium oxalate (CaOx) monohydrate crystals which is responsible for tissue damage in various organs. Another mechanism in CaOx supersaturation due to EG is the increase in oxalate excretion and decrease in calcium, magnesium and citrate excretion in the tubules, which in turn results in crystalluria and stone formation [9–11].

Myrtus communis (MC) L. is a myrtle species whose leaves have been used as a traditional folk remedy in the Mediterranean region and Anatolia. MC has been shown to have beneficial effects against diarrhea, esophagitis, streptozocin-induced diabetes, burn injury and pulmonary fibrosis due to its antioxidant effects [12–16]. Due to its proven benefits in the treatment of oxidative stress-related conditions, we examined the effects of MC L. extract in an EG-induced nephrolithiasis model in rats.

Highlight key points

- Nephrolithiasis is a kidney disease associated with oxidative damage.
- MC is an antioxidant plant.
- As a protective and therapeutic agent, MC reduces the formation of kidney stones.

MATERIALS AND METHODS

The collection and extraction of plant material was carried out according to the previous study performed by our team [15]. This study was approved by Marmara University Animal Experiments Local Ethics Committee with protocol number 30.2017.mar, dated 03.04.2017. The study design is in accordance with the Helsinki Declaration and conforms the Committee on Publication Ethics (COPE) guidelines.

Rats were supplied by the Marmara University Experimental Animals Application and Research Center. During the experiment, the rats were kept in rooms with 12-hour light-dark lighting, temperature of 20–22°C and humidity of 45–50%. During this period, all rats were kept in transparent cages and fed with standard rat chow ad libitum.

Groups and Induction of Nephrolithiasis

Thirty-two male Wistar albino rats, approximately 3 months old, weighing 250–300 g, were randomly divided into four groups (n=8/group).

- Control group (C): Rats were given normal drinking water for 8 weeks.
- Ethylene glycol group (EG): Rats were given drinking water containing 0.75% EG for 8 weeks.
- MC Prophylaxis group (MC+EG+MC): Daily MC extract was administered simultaneously with 0.75% EG at a dose of 100 mg/kg for 8 weeks.
- MC Treatment group (EG+MC): Rats were given drinking water containing 0.75% EG for 8 weeks. 4 weeks after the onset of EG, MC extract 100 mg/kg was started daily and given for the remaining last 4 weeks of the experiment.

At the conclusion of the study, the rats were placed in metabolic cages and 24-hour urine samples were collected. Thereafter, under ether anaesthesia, intracardiac blood samples were taken, animals were sacrificed and kidney tissues were harvested for histological and biochemical evaluations.

Table 1. Calcium, oxalate, citrate and creatinine levels in 24-hour urine samples

	Control	EG	MC+EG+MC	EG+MC
Calcium	0.48±0.03	0.24±0.03***	0.46±0.05^{++}	0.46±0.05^{+++}
Oxalate	0.57±0.06	1.88±0.16***	0.94±0.09^{+++}	1.22±0.15$^{*,++}$
Citrate	6.18±0.66	1.68±0.42***	4.27±0.53^{+}	3.16±0.45**
Creatinine	10.11±0.67	5.12±0.67***	8.68±0.87^{+}	5.51±0.67**

*: P<0.05; **: P<0.01; ***: P<0.001: vs control; +: P<0.05; ++: P<0.01; +++: P<0.001: vs EG group; EG: Ethylene glycol; MC: *Myrtus communis*.

Biochemical Measurements

Commercial kits were used for urinary calcium, citrate, oxalate, and creatinine measurements; QuantiChromTM Calcium Assay Kit (DICA-500), EnzyChromTM Oxalate Assay Kit ((EOXA-100), EnzyChromTM Citrate Assay Kit (ECIT-100), and Rat Creatinine (CR) ELISA Kit (MBS3807987), respectively.

Oxidative DNA Damage Measurement

Oxidative DNA damage in tissues was determined by measuring 8-Hydroxy-deoxyguanosine (8-OHdG) levels. Measurements were performed with the ELISA Kit according to kit manuals for the procedures. Results are expressed as 8-OHdG ng/mg DNA.

Determination of Myeloperoxidase Activity

Myeloperoxidase (MPO) activity was determined according to Hillegass et al.'s method [17]. The supernatant was discarded after the tissue homogenates were centrifuged for 10 minutes. 3 ml of 0.5% HETAB was added to the precipitate and homogenized, frozen 3 times, thawed and sonicated, and centrifuged to work with the upper phase. Afterwards, 50 mM K_2HPO_4 (pH: 6), o-dianisidin-2 HCl, and 20 mM H_2O_2 (Hydrogen Peroxide) solutions were added and the reaction was terminated by adding 2% sodium azide. Centrifugation was done for 10 minutes at 3000 rpm. The absorbance of the color formed by taking the supernatant was read in the spectrophotometer at 460 nm. The extinction coefficient for MPO was calculated as 42M-1cm^{-1}. The results were expressed as U/g protein.

Caspase-3 Activity Determination

Caspase-3 activity levels were determined using commercial kits (Caspase 3 ELISA Kit 96T). The measurement principle was based on the spectrophotometric assessment of the formation of chromophore p-nitroaniline (pNA) from the caspase-3 substrate N-Acetyl-Asp-Glu-Val-Asp p-nitroanilide (Ac-DEVD-pNA). The absorbance of the liberated pNA was found by reading at 405 nm using an ELISA reader.

Measuring Osteopontin Levels and N-acetyl-β-glucosaminidase (NAG) Enzyme Activity

Measurement of osteopontin levels and NAG enzyme activity in tissues were determined using the OPN ELISA Kit and NAG ELISA Kit, respectively. Results were expressed as ng/g for osteopontin, while NAG activity was expressed as U/mg protein.

Histological Analysis

Histopathological analyses were performed after kidney tissues were fixed in 10% formalin solution and dehydrated in degraded ethanol series and cleared in toluene. Paraffin-embedded samples were cut into 5 µm-thick sections using a rotary microtome and stained with hematoxylin and eosin. Sections were assessed and photographed using an Olympus BX51 light microscope (Olympus Co., Ltd., Tokyo, Japan).

Statistical Analysis

Statistical analyses were performed using GraphPad Prism 3.0 (GraphPad Software, San Diego, CA). Data are expressed as mean±standard error. For comparisons, analysis of variance followed by Tukey's multiple comparison tests were used. Values of $p<0.05$ were considered significant.

RESULTS

24 h urinary calcium level decreased significantly ($p<0.001$) in the EG group, and was increased back to the C group value in both MC+EG+MC and EG+MC groups (Table 1).

Table 2. Myeloperoxidase (MPO) and caspase-3 activities, N-acetyl-β-glucosaminidase (NAG), 8-hydroxydeoxyguanosine (8-OHdG) and osteopontin levels in control (C), ethylene glycol (EG), MC Prophylaxis group (MC+EG+MC), and MC treatment group (MC+EG+MC)

	C	EG	MC+EG+MC	EG+MC
MPO (U/g)	6.9±0.4	13.1±0.8***	7.2±0.7^{+++}	9.3±1.0^{+}
NAG (U/mg prot)	0.5±0.05	0.9±0.06***	0.3±0.03^{+++}	0.3±0.02^{+++}
8-OHdG (ng/mg DNA)	2.5±0.3	7.1±0.5***	4.4±0.4$^{**,+++}$	5.4±0.2$^{***,+}$
Caspase activity (nmolpNA/mg prot)	9.4±0.4	18.8±1.6***	9.1±1.1^{+++}	12.5±0.5^{++}
Osteopontin (ng/g)	1.6±0.1	2.8±0.09***	2.2±0.1$^{*,+}$	2.7±0.2***

***: P<0.001; **: P<0.01; *: P<0.05: versus control; +++: P<0.001; ++: P<0.01; +: P<0.05: versus EG group.

24 h urinary oxalate levels were increased significantly (p<0.001) in the EG group. Compared to EG group values, the 24 h urinary oxalate levels were significantly lower in both MC+EG+MC and EG+MC groups (p<0.001, p<0.01). The values in the MC+EG+MC group were similar to the C group but 24 h urinary oxalate levels, although lower than the EG group, still remained significantly higher than the C group (p<0.05) (Table 1).

24 h urinary citrate level was significantly decreased (p<0.001) in the EG group and increased significantly (p<0.05) in the MC+EG+MC group. However, in the EG+MC treatment group, although increased to some extent, the citrate level was still significantly lower (p<0.01) compared to the C group (Table 1).

24 h urinary creatinine level decreased significantly (p<0.001) in the EG group and increased significantly (p<0.05) in the MC+EG+MC treatment group. However, in the EG+MC group, 24 h urinary creatinine levels were still significantly lower (p<0.01) compared to the C group (Table 1).

The MPO activity increased significantly (p<0.001) in the EG group. Compared to the EG group, MPO levels decreased significantly in MC+EG+MC and EG+MC groups and were found to be similar to the Control group values (Fig. 1A, Table 2).

In the EG group, compared to Control values, NAG activity was increased significantly (p<0.001). Activity levels were decreased significantly (p<0.001) in both MC+EG+MC and EG+MC groups compared to the EG group (Fig 1B, Table 2).

In the EG group, Caspase-3 enzyme activity was increased significantly (p<0.001) compared to the C group. The activity level decreased significantly (p<0.01, p<0.001) in both MC+EG+MC and EG+MC groups compared to the EG group (Fig. 2A, Table 2).

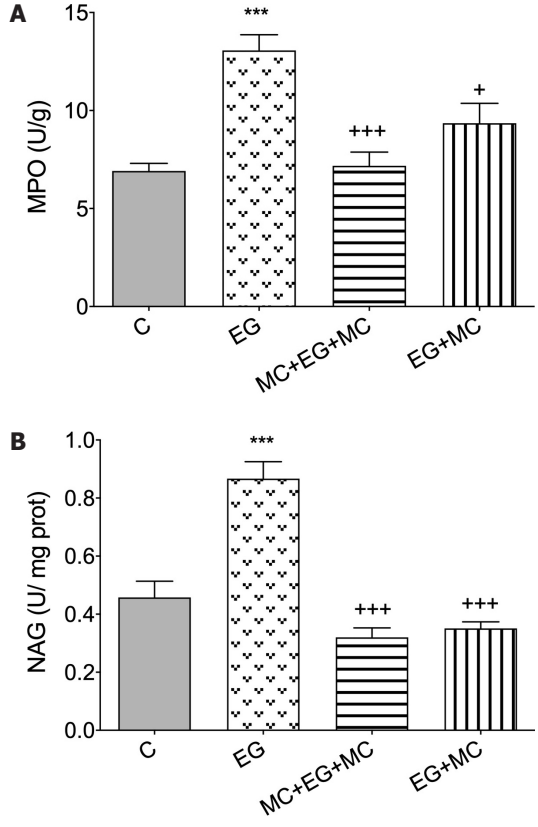

Figure 1. **(A)** Myeloperoxidase (MPO) and **(B)** N-acetyl-β-glucosaminidase (NAG) activities in the kidney tissues.

***: P<0.001: vs control group; +: P<0.05; +++: P<0.001: vs EG group.

In the EG group, the 8-OHdG level was increased significantly compared to the C group (p<0.001). MC+EG+MC group had significantly lower 8-OHdG levels compared to EG group (p<0.01). Although there was a significant (p<0.01) decrease in the EG+MC group compared to the EG group, the 8-OHdG level was still higher compared to the C group (Fig. 2B, Table 2).

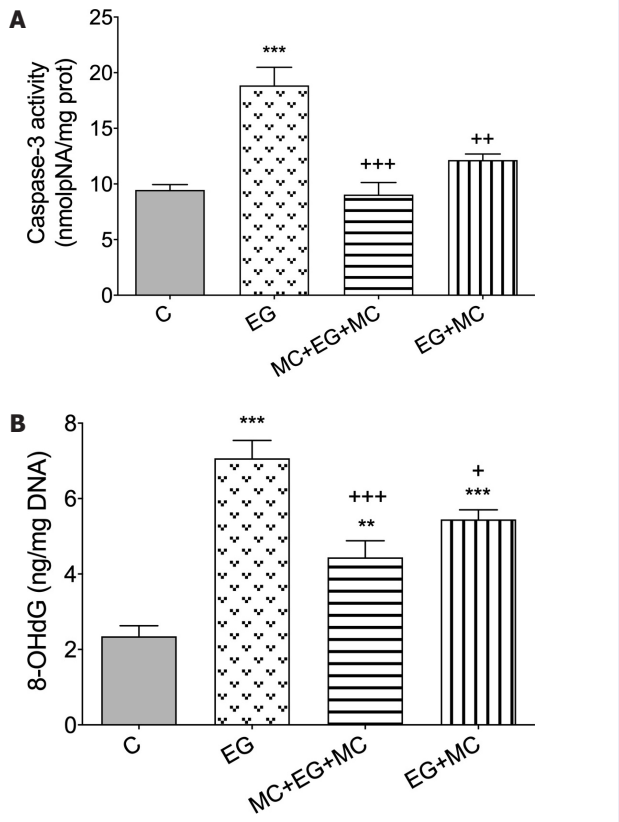

FIGURE 2. **(A)** Caspase-3 activity and **(B)** 8-hydroxydeoxyguanosine (8-OHdG) levels in the kidney tissues.

**: P<0.01; ***: P<0.001: vs control group; +: P<0.05; ++: P<0.01; +++: P<0.001: vs EG group.

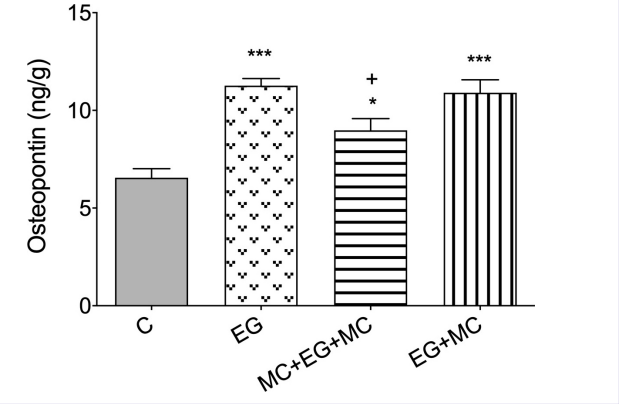

FIGURE 3. Osteopontin levels in the kidney tissues.

*: P<0.05; ***: P<0.001: vs control group; +: P<0.05: vs EG group.

Osteopontin levels were increased significantly in both the EG and EG+MC groups compared to the C group (p<0.001, p<0.001). In the MC+EG+MC group, osteopontin levels were significantly lower compared to the EG group (p<0.05) (Fig. 3, Table 2).

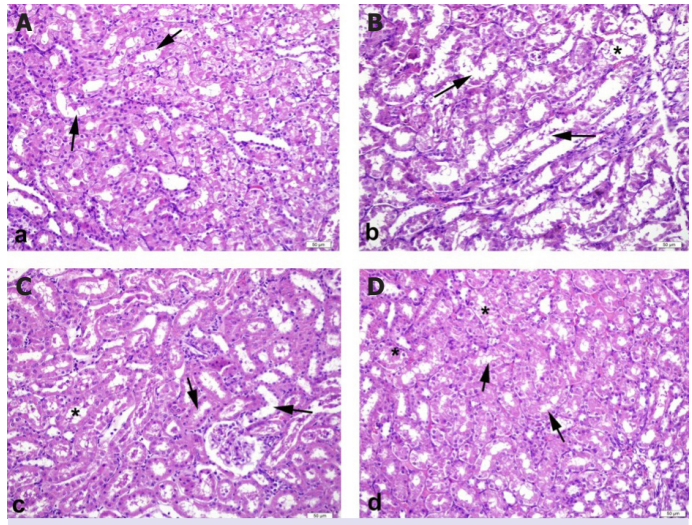

FIGURE 4. **(A)** Control group showing regular layout of tubular structures (arrows); **(B)** EG group showing severe degeneration with tubular basal membrane thickening, tubular cell desquamation (arrows), and luminal shredding (*); **(C)** MC + EG group showing normal-layout tubular basal membranes (arrows) and decreased tubular cell shredding (*); **(D)** EG + MC group showing reduced thickening of basal membranes (arrows) with slightly shredded tubular cells (*). Hematoxylin and Eosin staining, Magnification: X 200.

Histopathological examinations showed that the C group had regular glomerulus and tubular structures (Fig. 4A) while EG caused severe degeneration with tubular basal membrane thickening, tubular cell desquamation, and luminal shredding (Fig. 4B). In the MC+EG+MC group, the basal membrane thickening regressed and reduced tubular cell shredding was observed (Fig. 4C) and in the EG+MC group, slight basal membrane thickening and reduced tubular cell shredding were investigated (Fig. 4D).

DISCUSSION

Genus *Myrtus* L. is a member of the Myrtaceae family and, it is represented by a single species in Turkiye, which is called *Myrtus communis* L. subsp. *communis*. MC L. leaves, fruits and volatile oils are frequently used as folk remedies for several purposes [18]. MC has been previously shown to have various biological effects such as antioxidant, anti-diabetic, hypotensive, anti-inflammatory, antimicrobial, hepatoprotective, anti-hypercholesterolemic and anticancer activities [19–23]. In this study, the effects of MC in a nephrolithiasis model in rats were investigated.

Amin et al. [24] reported that while urinary citrate excretion decreased in the EG-induced nephrolithiasis model, prophylactic administration of 100 mg/kg of crocus sativus (saffron) extract significantly prevented the decrease in citrate levels on the 30th day. Low citrate level is an important risk factor for kidney stone formation [25]. In our study, we reported that the levels of citrate, an inhibitor of stone formation, decreased in the EG group, and increased in rats preemptively treated with the MC plant. Citrate levels were still decreased in the MC-treated group (EG+MC group) compared to the C group, so we can say that preventive treatment was more effective in preventing kidney stone formation compared to treatment after the stone-forming agent is given.

In a study by Hadjzadeh et al. [26] urinary oxalate concentration was found to be increased significantly in the EG-induced nephrolithiasis group compared to their Control group. The authors reported Nigella sativa plant extract significantly reduced the urinary oxalate concentration. Similarly, in another study by Ilbey et al. [27] 24 h urinary oxalate level that was increased in the EG-induced nephrolithiasis group was reversed back to the C group values upon treatment with medium and high doses of pomegranate juice. We had similar results in our study. Urinary oxalate levels were increased in the EG group, while they were decreased significantly in both MC+EG+MC and EG+MC groups.

Similar variations in 24 h urinary calcium and creatinine levels were observed upon pretreatment and treatment with MC in rats subjected to EG to induce nephrolithiasis.

EG administration is a commonly used model to create nephrolithiasis model in animal studies [9]. EG is metabolized to glycolate, glyoxylate, and oxalate, leading to calcium oxalate crystal deposition in both urine and kidneys. In order to create this model, drinking water containing 0.75% EG solution is administered to rats. Chronic hyperoxaluria caused by the administration of EG alone causes severe crystalluria and CaOx nephrolithiasis in subjects [28].

Osteopontin promotes cell adhesion, chemotaxis and signal transduction in various cell types and plays an important role in renal damage associated with inflammatory glomerulonephritis, obstructive uropathy, and tubular interstitial disease [29]. Renal OPN is secreted into the urine and directs CaOx crystals to the calcium-dihydrate phase. Calcium-dihydrate crystals adhere to kidney epithelial cells significantly less than calcium oxalate monohydrate crystals [30]. It has been shown in studies that osteoponin strongly inhibits the nucleation, growth and attachment of CaOx crystals to kidney epithelial cells. In nephrolithiasis models induced by glyoxylic acid, OPN has been shown to significantly increase mRNA and protein expression in in all segments of the renal tubules [29]. Sener et al. [9] reported that OPN gene expression increased significantly in the EG group and antioxidant melatonin treatment reversed this increase. Similarly, in our study, osteopontin levels increased in the tissue in EG-induced nephrolithiasis model. On the other hand, we reported that the OPN expression decreased in the MC+EG+MC group where MC was given as a preventive treatment approach.

MPO plays an important role in the antimicrobial and antiviral system and is found in the circulation of some tissue macrophages, including neutrophils, monocytes, and microglia. During the phagocytosis of pathogens, azurophilic granules release their contents together with MPO into phagolysosomes. It is known that inflammatory tissue damage is largely driven by MPO-derived oxidants [31]. The MPO activity is an indicator of inflammation and neutrophil infiltration in the tissues. In our study, the significant increase in MPO activity in the EG group indicates that tissue damage caused by EG is through an inflammatory pathway. On the other hand, both treatment groups with MC (MC+EG+MC and EG+MC groups as protective and after stone formation, respectively) had a significantly reduced MPO activity, which suggests that this extract protects kidney tissue upon its antioxidant and anti-inflammatory properties.

Caspase 3, 6 and 7 can be detected in apoptotic cells. Activated apoptotic caspases result in generation of signaling events that allow controlled destruction of cellular components [32]. It is known that oxidative reactions accompany apoptotic damages. Urinary excretion of 8-hydroxydeoxyguanosine (8-OHdG), an indicator of oxidative DNA damage, is significantly higher in patients with nephrolithiasis than in healthy individuals, indicating that these patients have a higher degree of oxidative stress [33]. In a clinical study, 8-OHdG expression was reported higher in renal tubular cells detected in renal biopsies of patients with nephrolithiasis, and increased leukocyte infiltration was observed in renal tissues compared to patients without nephrolithiasis [33]. In our study, oxidative damage caused by ethylene

glycol administration significantly increased caspase-3 activity and oxidative DNA damage (8-OHdG) by stimulating apoptosis in kidney tissue. These increases were significantly reversed in both MC-treated groups. In EG+MC group, where MC was started after four weeks of EG, although these parameters were decreased compared to EG group, results were still higher than Control group values. These results suggested that both prophylactic and therapeutic application of MC plant (MC+EG+MC and EG+MC, respectively) can prevent apoptosis; preventive treatment may be more effective in this sense.

NAG is a lysosomal enzyme found in proximal tubular cells and is an indicator of renal tubular function. When proximal tubular cells are damaged by any means, urinary NAG level increases. Therefore NAG measurements are used as a reflection of proximal tubular cell necrosis [34]. In one of our group's previous studies, we demonstrated that NAG activity is increased significantly EG-induced nephrolithiasis group. The antioxidant treatment with melatonin decreased the enzyme activity significantly compared to the EG group [9]. Similar to these previously mentioned data, in our study, NAG activity was increased significantly in the EG group compared to the C group activity. On the other hand, NAG activity was decreased in both the MC pre-treated group (MC+EG+MC group) and the MC-treated group (EG+MC group) compared to the EG group.

Conclusion

Nephrolithiasis, as a recurrent and frequent pathological condition, requires a meticulous preventive, medical and surgical approach. Unless well-treated medically and surgically, the recurrences and the unwanted end result, which is an end-stage renal disease, create a great socio-economical burden. Therefore, preventive measures are as important as treatment options. Due to the proven relations between nephrolithiasis and oxidative stress, anti-inflammatory and antioxidant agents can be very well suited for the basis of preventive measures.

MC extract, which has been proven effective against various pathologies due to its antioxidant properties, has been shown to be a promising protective and therapeutic option against the EG-induced nephrolithiasis model. Plant extracts such as MC can be suitable treatment adjuncts as over-the-counter supplements if supported by future clinical prospective studies.

Ethics Committee Approval: The Marmara University Animal Experiments Local Ethics Committee granted approval for this study (date: 03.04.2017, number: 30.2017.mar).

Authorship Contributions: Concept – BE, DD, OG, AS, OC, SC, PE, AA, TES, GS; Design – BE, DD, OG, AS, OC, SC, PE, AA, TES, GS; Supervision – BE, DD, OG, AS, OC, SC, PE, AA, TES, GS; Fundings – BE, OG, AA, GS; Materials – OG, AS; Data collection and/or processing – BE, OG, OC, SC, PE, TES, GS; Analysis and/or interpretation – BE, TES, GS; Literature review – BE, DD, OG; Writing – BE, DD, TES; Critical review – TES, GS.

Conflict of Interest: No conflict of interest was declared by the authors.

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