

Full Proceeding Paper

SALUBRIOUS EFFECT OF ROTTLELIN ON HYPEROXALURIA INDUCED OXIDATIVE DAMAGE IN RATS

NIRLEP CHHIBER¹, TANZEER KAUR², SURINDER SINGLA^{1*}

¹Department of Biochemistry, Panjab University, Chandigarh 160015, India, ²Department of Biophysics, Panjab University, Chandigarh 160015, India

Email: singlask6@gmail.com

Received: 26 Nov 2015 Revised and Accepted: 12 Feb 2016

ABSTRACT

Objective: To investigate the *in vitro* oxidant scavenging properties of rottlerin and to study the potential role of rottlerin on ethylene glycol induced nephrocalcinosis in rats.

Methods: *In vitro* oxidant scavenging properties of rottlerin were studied along with its effect on *in vitro* calcium phosphate mineralization. For the *in vivo* studies, hyperoxaluria was induced by administering 0.4 % ethylene glycol and 1 % ammonium chloride in drinking water to male wistar rats for 9 d. Rottlerin was administered intraperitoneally at 1mg/kg/d along with the hyperoxaluric agent. Total thiols content, activities of glutathione-S-transferase (GST), glutathione reductase (GR), Citrate synthase (CS), isocitrate dehydrogenase (ICDH), ATPase and urinary parameters were studied.

Results: Rottlerin showed *in vitro* DPPH, superoxide, and ABTS radical scavenging activity along with inhibition of calcium phosphate mineralization in an *in vitro* homogeneous system. The diminished activities of GST, GR, ICDH, CS, ATPase and level of total thiols were considerably stabilized by rottlerin, suggesting that rottlerin provides protection against oxalate induced oxidative damage.

Conclusion: We suggest that rottlerin protects the integrity of the renal cell by stabilizing the free-radical mediated damage. Thus, the present study reveals that the antioxidant nature of rottlerin protects the renal cells against oxalate-induced injury and thus, rottlerin may prevent against hyperoxaluria induced oxidative damage.

Keywords: Rottlerin, Hyperoxaluria, Oxidative stress, Antioxidant

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INTRODUCTION

Hyperoxaluria is one of the major risk factors for calcium oxalate kidney stone formation in humans. Oxalate is normally excreted by the kidneys, however, increased urinary excretion of oxalate, can be toxic largely because of its propensity to crystallize at physiologic pH and form calcium oxalate crystal deposits in the kidneys [1]. In the kidneys, calcium oxalate crystals can block the renal tubules, disrupt cellular functions and kill nearby cells. Despite the major technical achievements for stone removal in the last three decades, the problem of recurrent stone formation remains. The recurrence rate of kidney stones is approximately 15 % in the first year and as high as 50 % within 5 y of the initial stone [2].

Oxalate and calcium oxalate crystals induced oxidative stress leads to renal injury and inflammation. Antioxidants vitamin E, catalase, deferoxamine, superoxide dismutase, and its mimetic provide protection from the generation of ROS and associated lipid peroxidation and injury. Citrate is also involved in maintaining endogenous antioxidant defenses [3].

Rottlerin, unlike other polyphenols, is not present in edible vegetables and in common beverages; instead, it is primarily present in the gland hair covering the fruit of *Mallotus philippinensis* (Euphorbiaceae), that is inedible and only used by indigenous populations of Southeast Asian tropical regions. Rottlerin is used as a dye for coloring textiles and as an old folk remedy against tapeworm (when taken orally) and scabies and ringworm (when applied topically) [4]. It inhibits PKC- δ (IC₅₀=3-6 μ M), PKC- α , β , γ (IC₅₀=30-42 μ M) and PKC ϵ , η (IC₅₀=80-100 μ M). It also inhibits p38 regulated activated protein kinase; MAPK activated protein kinase, JNK α 1, cAMP-dependent protein kinase 2, JNK α 1, cAMP-dependent protein kinase, 3-phosphoinositide dependent protein kinase 1, protein kinase B- α , GSK-3- β along

with non-kinase enzymes like β -lactamase, α -chymotrypsin and malate dehydrogenase [5].

Two molecules of DPPH are reduced by one molecule of rottlerin, indicating that rottlerin can donate two hydrogens from its five phenolic hydroxyl groups. The antioxidant properties of rottlerin have also been confirmed in cultured cells (MCF-7) [6-7]. Rottlerin exhibits anti-inflammatory properties by inhibiting arachidonic acid metabolism through some pathways, such as interference with PKC- δ signaling, inhibition of NF- κ B and STAT transcriptional activity, and/or direct binding to COX and LOX [5].

The supplementation of vitamin E, methionine, and lipoic acid was able to normalize the antioxidant status, leading to the prevention of renal injury and thereby associating a role for antioxidants in the pathophysiology of oxalate-mediated injury and calcium oxalate deposition [8-10]. These data maintain the notion that rottlerin may protect renal cells against injury induced by oxalate mediated oxidative stress. In the light of antioxidant and anti-inflammatory properties of rottlerin, the present study explored the effect of rottlerin on mineralization and hyperoxaluric manifestations in rats. To date, however, no study about this presumed effect has been reported.

MATERIALS AND METHODS

Chemicals

All the chemicals used were of analytical grade and were purchased from Sigma Chemical Co. (St. Louis, USA), Merck (Mumbai, India) and Sisco Research Laboratories Pvt. Ltd. (Mumbai, India). Rottlerin was purchased from Calbiochem, Germany.

In vitro oxidant scavenging activity

The oxidant scavenging activity using DPPH was assayed by the method as stated by Shen *et al.*, 2010 [11]. Superoxide radical scavenging activity was assayed by the method of Rao and

Kunchandy, 1990 [12]. ABTS scavenging activity was estimated by the method as stated by Sithisarn *et al.* 2005 [13]. Ascorbic acid was used as a control and the results were expressed as percentage inhibition with respect to control.

In vitro mineralization assay

To determine the extent of calcium phosphate (CaP) precipitation, homogenous mineralization system was used to study the extent of *in vitro* mineral phase formation in the absence of any matrix. The homogenous system consisted of 0.1 M Tris buffer (pH 7.4), 5 mM CaCl₂ and 5 mM KH₂PO₄. After incubating this system at 37 °C, precipitates obtained were centrifuged, and the pellets were resuspended in 0.1N HCl. This 5 ml system for mineralization, already standardized in our laboratory was used to study the extent of *in vitro* mineral phase formation in the absence of any matrix [14-15]. The calcium and phosphate ions concentration in the precipitate represented the extent of precipitation of these ions and the sample containing inhibitory biomolecule(s) minimized the extent of their precipitation. The percent inhibition in the mineral phase was calculated as % inhibition = [(C-T)/C] x 100, where C is the concentration of calcium and phosphate ions of the precipitate formed in the control system and T are the concentrations of calcium and phosphate ions of the precipitate formed in the test system, respectively. The concentration of calcium and phosphate ions was measured [16-17].

Animals and treatment schedule

Healthy male wistar rats weighing between 150 and 200 g of equivalent age groups were obtained from the central animal house of Panjab University, Chandigarh, India. The procedures followed were approved by the Institutional Animal Ethics Committee and were in accordance with the Guidelines for Humane Use and Care of Laboratory Animals (PU/IAEC/S/14/41).

To induce CaOx crystal formation, rats were exposed to 0.4 % ethylene glycol (EG) with 1.0 % ammonium chloride (NH₄Cl) in drinking water for 9 d. All rats were randomly divided into the groups having 5-7 rats each. Normal (NRM) rats were provided with standard animal feed and water ad libitum for 9 d. Hyperoxaluric group (HYO) of rats were given 0.4 % EG (v/v) with 1.0 % NH₄Cl (w/v) in drinking water for 9 d. rottlerin treated HYR1 group was administered an intraperitoneal dose of 1mg/kg/day rottlerin. In addition to the hyperoxaluric dose of 0.4 % EG with 1.0 % NH₄Cl in their drinking water for 9 d. ROT1 group rats were given an intraperitoneal dose of 1mg/kg/d rottlerin alone for 9 d. The standardization of the hyperoxaluric rat model was already done in the lab from previous studies [18].

Sample collection

At the end of treatment period, rats were placed in metabolic cages, and urine was collected. The concentration of phosphate was

estimated in urine [17]. Rats were anesthetized with diethyl ether and sacrificed by decapitation on day 10.

Isolation of mitochondria

The kidney was washed in normal saline at 4 °C, trimmed of adipose and connective tissues, weighed, and homogenized, (10 % w/v) in buffer containing 0.25 M sucrose, 5 mM HEPES, 1 mM EDTA, and 0.1 % bovine serum albumin pH 7.2. The homogenate was centrifuged at 1000x g for 5 min to remove the nuclear fraction and cell debris. Mitochondrial pellet was obtained by centrifuging the post-nuclear supernatant at 14,000x g for 20 min. The pellet was washed thrice with 1.15 % potassium chloride solution and finally suspended in 0.25 M sucrose solution. The mitochondrial fraction was used for various assays.

Measurement of antioxidant status

Total thiols (T-SH) were determined in the mitochondria and homogenate according to the method of Sedlak [19]. The results were expressed as nmol T-SH/mg protein. The activity of isocitrate dehydrogenase (ICDH) in was determined by the method of Bergmeyer and Bernt [20]. The results were expressed as nanomoles of NADPH formed/min/mg protein. Glutathione reductase activity in post-mitochondrial fraction was assayed by the method of Carlberg and Mannervik [21]. Enzyme activity was calculated using the molar extinction coefficient of NADPH. The results were expressed as nanomoles of NADPH oxidized/min/mg protein. Glutathione-S-transferase (GST) activity in post-mitochondrial fraction was assayed in the post-mitochondrial supernatant by the method of Habig *et al.* [22]. Mitochondrial ATPase activity was assayed as described by Griffiths and Houghton [23]. Citrate synthase (CS) was assayed by the method of Spinazzi *et al.* [24]. The results were expressed as nanomoles of TNB formed/min/mg protein.

Statistical analysis

Data were analyzed by one-way ANOVA and the Tukey's test for multiple comparisons using GraphPad Prism (version 5.0; San Diego, USA). They are expressed as mean±SD. Results were considered significant if P < 0.05.

RESULTS

In vitro antioxidant activity of rottlerin

The *in vitro* antioxidant activity of rottlerin was assayed using three different activity assays. DPPH radical scavenging activity assay with respect to ascorbic acid revealed that the maximum percentage inhibition of DPPH radical was seen at a concentration of 40 µg (97.82 %) and at 80 µg (56.35 %) and 100 µg (46.03 %) the inhibitory activity of rottlerin rapidly declined (Fig.1A). Superoxide radical inhibition of rottlerin was found to be highest at a concentration of 40 µg (69.29 %) with respect to ascorbic acid (Fig.1B). At a concentration of 60 to 100 µg the increase in the inhibition was proportional to the increase in the concentration of rottlerin. ABTS radical scavenging activity was found to be increased with an increase in the concentration of rottlerin with respect to ascorbic acid (fig. 1C).

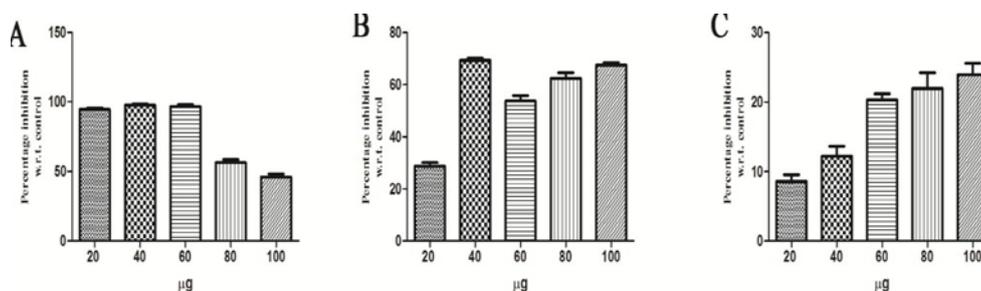


Fig. 1: In vitro radical scavenging activity of rottlerin. A) DPPH radical scavenging activity assay B) Superoxide radical scavenging activity assay C) ABTS radical scavenging activity assay. The results are expressed as percentage inhibition with respect to ascorbic acid. Values are expressed as mean±SD of three similar experiments (n=3)

Effect of rottlerin on in vitro calcium phosphate mineralization

To evaluate the effect of rottlerin on the initial calcium phosphate mineral phase formation, the extent of inhibition of calcium and

phosphate ions was measured. It was found that the maximum inhibition of calcium ions was at a concentration of 1 mg of rottlerin (49.33 %). The inhibition of calcium ions are increased with an increase in the concentration of rottlerin (fig. 2B). A similar trend

was observed in the inhibition of phosphate ions. The maximum inhibition was noted at a concentration of 1 mg of rottlerin (75.63 %). The percentage inhibition observed was with respect to that of a control system (fig. 2B).

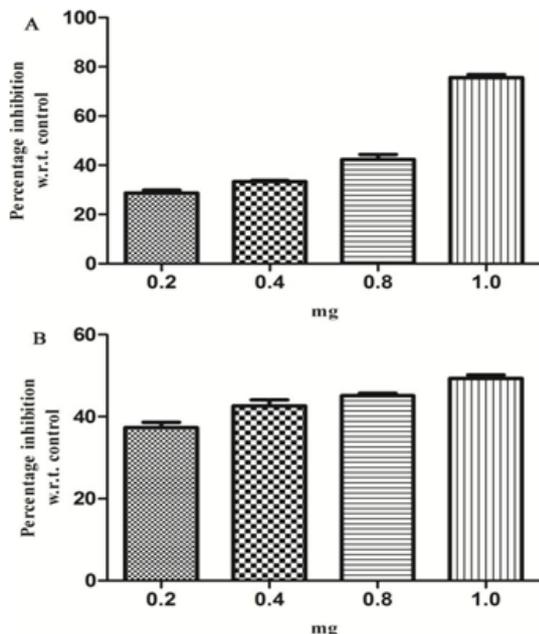


Fig. 2: Inhibition of *in vitro* calcium phosphate mineral phase by rottlerin A) Inhibition of calcium ions B) Inhibition of phosphate ions. The results are expressed as percentage inhibition with respect to control. Values are expressed as mean±SD of three similar experiments (n=3)

Effect of rottlerin on kidney to body weight ratio, urinary pH, and urinary phosphate

Kidney to body weight ratio was found to be increased in the HYO rats as compared to NRM group. The rats treated with rottlerin showed a near control level of the ratio of the kidney to body weight. Urine of HYO rats showed an acidic pH as compared to the NRM

group (fig. 3A). Rottlerin treated rats showed pH values similar to those of NRM rats (fig. 3B).

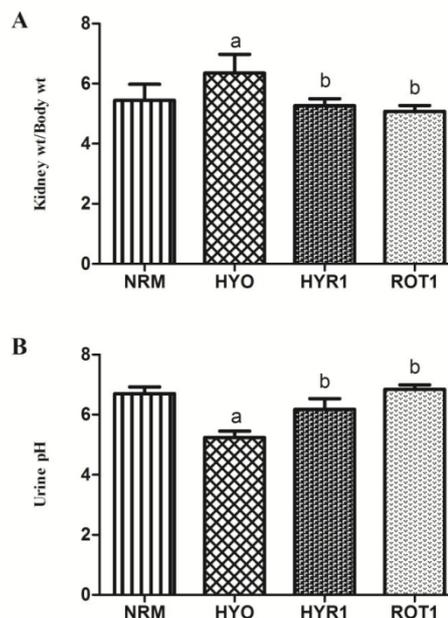


Fig. 3: Effect of rottlerin on urinary parameters in hyperoxaluric rats A) Kidney weight/Body weight B) Urinary pH. NRM: normal rats; HYO: hyperoxaluric rats; HYR1: hyperoxaluric rats treated with rottlerin 1mg/kg/d; ROT1: normal rats treated with rottlerin 1mg/kg/d. Values are expressed as Mean±SD of 5 rats per group. The results are compared by ANOVA with Tukey's multiple comparison post hoc test; a: P<0.05 significantly different from NRM group; b: P<0.05 significantly different from HYO group

Urine of HYO group rats showed a significant increase in the concentration of phosphate (63.57 %) as compared to NRM rats. HYR1 rats showed a significant decline in urinary phosphate as compared to HYO rats (table1).

Table 1: Effect of rottlerin on A) Total thiols ; B) Glutathione-S-transferase; C) Glutathione reductase; and D) Urinary phosphate in hyperoxaluric rats.

	NRM	HYO	HYR1	ROT1
A) Total thiols (nmoles/ mg protein)	4.393±0.5428	2.790±0.6230 ^a (-36.48 %)	4.558±0.8639 (+63.36 %)	5.118±0.8300 ^b (83.44 %)
B) GST (nmoles of GSH-CDNB conjugate formed/min/mg protein)	14.14±0.2596	7.784±0.1534 ^a (-44.95 %)	10.47±0.1146 (+34.50 %)	12.270±0.1838 (+57.63 %)
C) GR (nmoles of NADPH oxidised/min/mg protein)	14.18±0.2633	7.289±0.1252 ^a (-48.59 %)	11.95±0.4559 (+63.94 %)	12.725±0.2847 (+74.57 %)
D) Urinary phosphate (mg/dl)	0.1768±0.020	0.2892±0.021 ^a (63.57 %)	0.2272±0.004 ^b (-21.43 %)	0.1233±0.024 ^b (-57.71 %)

NRM: normal rats; HYO: hyperoxaluric rats; HYR1: hyperoxaluric rats treated with rottlerin 1mg/kg/d; ROT1: normal rats treated with rottlerin 1mg/kg/d. Values are expressed as Mean±SD of 5 rats per group. The results are compared by ANOVA with Tukey's multiple comparison post hoc test; a: P<0.05 significantly different from NRM group; b: P<0.05 significantly different from HYO group.

Effect of rottlerin on total thiols, GST, and GR

To evaluate the effect of rottlerin on antioxidant status of HYO rats, the level of total thiols was estimated in mitochondrial fraction and post-mitochondrial supernatant. The results revealed a significant decline in the level of total thiols in the HYO rats in both the

fractions. Supplementation of rottlerin led to the restoration of total thiols content.

Furthermore, the activities of GST and GR significantly dwindled in the HYO rats. Rottlerin treatment at a dose of 1 mg/kg increased the activities of GST and GR by 34.50 % and 63.94 %, respectively (Table1).

Effect of rottlerin on activity of ATPase, isocitrate dehydrogenase, and citrate synthase

Activities of ATPase, ICDH, and CS were significantly diminished in the HYO group.

Rottlerin supplementation significantly restored the activities of ATPase and ICDH at the dose of 1mg/kg/d. However, the increase in activity of CS in HYR1 group was not significant as compared to the HYO group (fig.4 A, B, C).

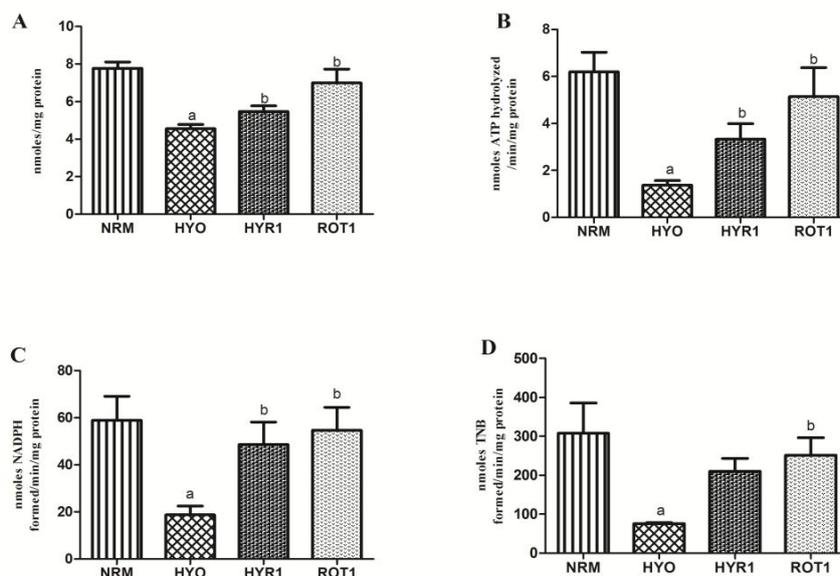


Fig. 4: Effect of rottlerin on A) Mitochondrial total thiols; B) ATPase; C) Isocitrate dehydrogenase; and D) Citrate synthase in hyperoxaluric rats. NRM: normal rats; HYO: hyperoxaluric rats; HYR1: hyperoxaluric rats treated with rottlerin 1mg/kg/d; ROT1: normal rats treated with rottlerin 1mg/kg/d. Values are expressed as Mean±SD of 5 rats per group. The results are compared by ANOVA with Tukey's multiple comparison post hoc test; a: P<0.05 significantly different from NRM group; b: P<0.05 significantly different from HYO group.

DISCUSSION

The current treatment of urolithiasis employs the use of minimally invasive and non-invasive stone managements such as extracorporeal shock wave lithotripsy, percutaneous nephrolithotomy, and endoscopy for the removal of kidney stones; however, high recurrence rates have been reported: 10 to 23 % per year [25] and 50 % at 5 y [26]. The problem of stone occurrence is one of the major concerns in urolithiasis therapy and, therefore, requires an effective prophylactic regimen. Potassium citrate therapy is recommended as a remedy intended to stretch the stone-free condition [27]. Kidney stone formation is a complex process and the result of a cascade of events, including crystal nucleation, growth, aggregation, and crystal retention within the renal tubules [28-30]. It has been proposed that oxalate-induced injury to renal tubular epithelial cells is caused by the production of reactive oxygen species. Oxidative stress influences cell injury and inflammatory processes that promote aggregation and retention of CaOx crystals [31]. Another study reported that renal tubular cell injury is mediated by free radical formation. High concentrations of oxalate, as well as CaOx crystals, are toxic to renal tubular cells [32].

Flavonoids are semi-essential food components that are ubiquitously present in nature. Natural products with a source of flavonoids are fruits, vegetables, grains, bark, roots, stems, flowers, tea, and wine. Free radicals attract various inflammatory mediators, contributing to a general inflammatory response and tissue damage. Flavonoids interfere with various free radical-producing systems including direct radical scavenging, reduction in ischemia-reperfusion injury by interfering with inducible nitric-oxide synthase activity, leukocyte immobilization during ischemia, inflammation conditions, and the xanthine oxidase pathway, which is an important oxidative injury route that is inhibited by flavonoids [33]. In an earlier report, the antioxidative effects of flavonoids (catechin) in green tea decreased oxidative injury in renal tubular cells and CaOx deposition in the rat kidney [34]. *In vitro* and *in vivo* studies have concluded that the bioflavonoid quercetin has potential antioxidant effects of decreasing the lipid peroxidation production induced by oxalate in MDCK cells [35]. Renal tubular damage is

associated with a decline in antioxidant defense of kidney and a decrease in the activity of free radical scavengers [36].

Reducing power is correlated with antioxidant activity and may serve as a significant reflection of the antioxidant activity. Compounds with reducing power indicate that they are electron donors and can reduce the oxidized intermediates of lipid peroxidation processes so that they can act as primary and secondary antioxidants [37]. Rottlerin is a flavonoid plant product isolated from the fruit covering of *Mallotus Phillipinensis* [4]. *In vitro* antioxidant activity assays demonstrated the antioxidant potential of rottlerin, which may be due to the presence of five-OH groups in its structure, which may act as scavengers of free radicals. Rottlerin effectively scavenged the DPPH, ABTS, and superoxide radicals when compared with respect to ascorbic acid. Furthermore, an *in vitro* calcium phosphate mineralization system developed in our laboratory showed the inhibitory potential of rottlerin on the extent of calcium and phosphate mineralization. It is well documented that calcium oxalate crystals grow attached to calcium phosphate crystals [38]. Inhibition of calcium phosphate mineralization may point towards its efficacy in inhibiting calcium oxalate crystallization.

Rat models of calcium oxalate urolithiasis induced by either ethylene glycol alone or in combination with other drugs such as ammonium chloride are often used to study the pathogenesis of kidney crystal deposition. In the light of inhibitory action on calcium phosphate mineralization and free radical scavenging properties, rottlerin was checked for its effects on a rat model of hyperoxaluria already well-established in our laboratory [18]. The biochemical mechanisms for this process are related to an increase in the urinary concentration of oxalate. In the present study, urinary pH was significantly decreased in the hyperoxaluric rats. The concomitant increase in urinary pH with rottlerin treatment suggested an increase in urinary citrate excretion. Citrate reduces CaOx supersaturation by the formation of complexes with calcium and directly inhibits crystal growth and aggregation by increasing urinary pH. Intracellular pH is altered by a change in systemic pH, leading to changes in citrate reabsorption and hence urinary citrate excretion [39]. The

significant increase in urinary pH of hyperoxaluric rats administered with rottlerin suggests its possible involvement in modifying kidney function. The kidney to body weight ratio of hyperoxaluric rats was significantly increased which suggested a loss of overall body weight of rats; rottlerin administration restored the kidney to body weight ratio to control levels. There was a significant increase in urinary excretion of phosphate in hyperoxaluric rats which suggested that increased urinary phosphate excretion along with hyperoxaluria provides an amiable environment for calcium oxalate crystal deposition as calcium phosphate crystals epitaxially induce calcium oxalate deposition [40]. However, rottlerin treatment reduced the risk of crystal deposition by lowering the urinary excretion of phosphate.

Restoration of the antioxidant defense system of cells reverses the oxalate and/or calcium oxalate crystal-induced renal damage and prevents additional crystal deposition [36]. Total thiols level was significantly diminished in hyperoxaluric rats as compared to the control groups along with a decrease in the activities of GR and GST. GR is crucial in maintaining the level of reduced glutathione by reduction of glutathione disulfide. GST is involved in detoxification of lipid peroxides by conjugation of reduced glutathione. As these enzymes are involved in the antioxidant defense of cell, the significant decline in the activities of these glutathione metabolizing enzymes suggests perturbation of oxidant/antioxidant balance in the renal cell milieu. The decrease in GR might be due to the reduction in the availability of its cofactor, NADPH [41]. Administration of rottlerin restored the activities and hence, antioxidant defense by these enzymes.

Mitochondria are one of the major targets of hyperoxaluria-induced oxidative stress. Hyperoxaluric group rats exhibited a significant decline in the level of total mitochondrial thiols suggesting a dwindled reductive environment in mitochondria. Likewise, the activity of mitochondrial enzymes such as ATPase, ICDH as well as that of CS was significantly lowered in the hyperoxaluric rats. Increase in oxalate load might be the possible reason for the dwindling activities of these enzymes, as reactive oxygen species produced by oxalate/calcium oxalate can inactivate the mitochondrial enzymes [41]. Treatment with rottlerin imparted protection to mitochondria against oxidative damage by restoring total thiols level to near control levels. The activities of ATPase and ICDH, but not of CS, were significantly restored by rottlerin treatment.

CONCLUSION

Taken together, the present study has implicated that free radical scavenging and restoration of antioxidant defenses protect renal cells from renal injury. Rottlerin exhibited *in vitro* radical scavenging activity as well as *in vitro* inhibition of calcium phosphate mineralization. It may be put forward that rottlerin is effective against ethylene glycol induced renal cell impairment by protecting the antioxidant defense systems in the renal tissue. The present study also demonstrated the protective effect of rottlerin against hyperoxaluria-induced oxidative damage in mitochondria. Positive effects of rottlerin in combating free radical damage in the biological system should be evident when inspected on a long-standing basis as free radical damage occurs in due course. The prospect of surmounting this anomaly with a range of modulators is a productive area for future exploration.

ACKNOWLEDGEMENT

The financial assistance provided by University Grants Commission, New Delhi is gratefully acknowledged.

CONFLICT OF INTERESTS

The authors state no conflict of interest.

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