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Biebersteinia multifida modulates diazinon-induced renal oxidative stress and ERK/p-ERK expression in rats

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Abstract

Diazinon (DZN) is an insecticide with a wide range of applications in developing countries, which can cause kidney toxicity through oxidative stress. *Biebersteinia multifida* DC (BM) is a medicinal plant native to Iran with reported anti-oxidant properties. This study examined the effect of BM root extract and DZN on extracellular signal-regulated kinase (ERK) and phosphorylated-ERK (p-ERK) expression levels and the activity of oxidative stress factors in a rat kidney. In this experimental study, 24 male rats were randomly divided into four groups, including control, DZN (40.00 mg kg^{-1}), BM (150 mg kg^{-1}), and DZN + BM. Treatments were administered orally once daily for 6 weeks. Kidney tissues were collected for biochemical assays, western blot analysis, and histological evaluation. Data were analyzed using SPSS Software. The DZN exposure induced marked renal injury, characterized by reduced glomerular mesangial matrix, thinning of the glomerular basement membrane, decreased laminin expression, and down-regulation of ERK and p-ERK proteins. Also, DZN altered anti-oxidant enzymes activities. In contrast, BM treatment improved histological parameters, restored laminin expression, and increased ERK and p-ERK levels. The BM also modulated oxidative stress markers by enhancing anti-oxidant enzymes activities and reducing oxidative imbalance. *Biebersteinia multifida* may exert protective effects against DZN-induced renal injury through improvement of glomerular structure, modulation of ERK signaling, and attenuation of oxidative stress, suggesting its potential nephroprotective properties.

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Introduction

Organophosphorus (OP) compounds are extensively used in developing countries, and their widespread application has come at a considerable cost to public health and environmental safety. Even chronic exposure to low doses of these pesticides produces adverse effects in humans and other animals over time.[1] Diazinon (DZN), an OP insecticide with broad-spectrum activity, is among the most extensively used pesticides in agriculture and animal husbandry worldwide, particularly in Iran.[2] Its toxicity is primarily mediated through irreversible inhibition of acetylcholinesterase and the induction of oxidative stress (OS), leading to damage in multiple organs. Given that DZN and its metabolites are predominantly excreted *via* the kidneys, renal tissue represents a major target of its toxic effects.[3]

Renal structure and function are critically dependent on the activation or deactivation of intracellular signaling pathways,[4] among which the mitogen-activated protein kinase (MAPK) pathway plays a particularly important role.[5] This pathway consists of a hierarchical kinase module, involving MAPK kinase kinase (*e.g.*, Raf-1 and B-Raf), MAPK kinase, and MAPK (extracellular signal-regulated kinase [ERK] 1/2), being activated sequentially through phospho-rylation events.[6] Although ERK is classically activated by

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mitogenic stimuli, accumulating evidence indicates that OS can also trigger ERK signaling, thereby influencing cell survival, proliferation, and injury responses.[7,8]

Oxidative stress has been shown to stimulate phosphorylation of Raf-1, MAPK kinase /2, and ERK1/2, leading to increased expression of pro-fibrotic and pro-angiogenic factors, such as transforming growth factor-β1 and vascular endothelial growth factor. Concurrently, activation of the renin–angiotensin–aldosterone system and phospholipase A2 contributes to the renal fibroblast activation, extracellular matrix (ECM) deposition, and mesangial cell proliferation, ultimately promoting progressive kidney damage.[4] Despite these findings, the involvement of ERK signaling in DZN-induced renal injury and its modulation by natural anti-oxidants remains insufficiently understood.

Given the central role of OS in OP-induced toxicity, medicinal plants with anti-oxidant properties have attracted growing interest as protective agents against chemically induced poisoning.[9] *Biebersteinia multifida* DC (BM), a plant traditionally used in various regions of Iran for the treatment of rheumatic disorders and bone-related conditions,[10,11] has demonstrated significant anti-oxidant[9] and anti-inflammatory activities.[12] Its bio-active constituents include flavonoids, alkaloids, terpenoids, sterols, and essential oils, which may contribute to its pharmacological effects.[11] Therefore, according to the mentioned cases, this experimental study aimed to investigate the effect of methanolic extract of BM root on ERK and phosphorylated-ERK (p-ERK) proteins, as well as on OS markers, in DZN-induced kidney injury in male Wistar rats.

Materials and Methods

Chemicals. Diazinon was purchased from Karon Co., Ltd (Hangzhou, China), Tris-HCl, ethylenediaminetetra-acetic acid, β-glycerophosphate, sodium deoxy-cholate, phenylmethylsulfonyl fluoride, sodium dodecyl sulfate, β-mercaptoethanol, and glycerol were purchased from Sigma-Aldrich (St. Louis, USA). Ketamine and xylazine were supplied by Alfasan Pharmaceutical Co. (Woerden, The Netherlands). Primary antibody as anti-Laminin (ab11575) and secondary antibody (ab205718) were purchased from Abcam (Cambridge, USA). All chemicals and reagents used for the biochemical assessment of antioxidant enzyme activities were obtained from Sigma-Aldrich.

Plant and extract. Roots of BM were collected and authenticated by Dr. Bahmanyar, a taxonomist at the Medicinal Plant Research Center, North Khorasan University of Medical Sciences, Bojnord, Iran. A voucher specimen (No. npmp57) was deposited at the herbarium of the center. The dried roots were powdered and extracted using a maceration method. Briefly, 200 g of plant powder was soaked in 1,000 mL methanol for 72 hr at room temperature with occasional stirring. The extract was then filtered and concentrated using a rotary evaporator (Laborota 4003, Heidolph, Schwabach, Germany) under reduced pressure. The resulting extract was dried at 40.00 ˚C and stored at 4.00 ˚C until use.[13] The extraction yield was 20.00% (w/w), corresponding to 40.00 g of dried extract obtained from 200 g of initial plant material.

Animal model and experimental design. A total of 24 male Wistar rats (8 weeks old and 220 - 250 g) were used. Animals were randomly assigned into experimental groups (n = 6): Control (no intervention), DZN (40.00 mg kg^{-1}; PO), BM (150 mg kg^{-1}; PO), and DZN (40.00 mg kg^{-1}; PO) + BM (150 mg kg^{-1}; PO). The rats were kept under standard conditions of temperature, humidity, water, and food. All experimental protocols were conducted in accordance with ARRIVE guidelines and approved by the Ethics Committee of the Mashhad University of Medical Sciences, Mashhad, Iran (IR.MUMS.AEC.1401.097). The selected dose of DZN (40.00 mg kg^{-1}; dissolved in ethanol) was based on previous studies, demonstrating subchronic nephrotoxicity without mortality.[14] The BM dose (150 mg kg^{-1}; dissolved in normal saline) was chosen according to the earlier pharmacological studies, reporting anti-oxidant and protective effects.[13] A separate vehicle control group was not included; previous studies using the same experimental conditions showed no significant effect of ethanol at the administered volume. The DZN and BM were administered by daily oral gavage to rats (for 6 weeks). At the end of the study, the animals were euthanized with the ketamine + xylazine (100+10.00 mg kg^{-1}; intraperitoneally), after which the right kidney was excised for measurement of OS factors and western blot analysis, while the left kidney was removed for histological examination.

Renal histology. Kidney tissues were fixed in 10.00% formalin, dehydrated, and embedded in paraffin. Sections (5.00 µm) were prepared and stained using periodic acid-Schiff (PAS) and Jones methenamine silver (JMS) methods according to the standard protocols. Histological evaluation was performed using light microscopy, and quantitative analysis was conducted using ImageJ Software (National Institutes of Health, Bethesda, USA).[15,16] Mesangial index was calculated as follows:

$$\text{Mesangial index} = \frac{\text{Area of PAS staining}}{\text{Total area of glomerulus}} \times 100$$

Immunohistochemistry. The basis of this method is antibody-antigen reaction. The immunohistochemistry staining was performed according to the Rahimi Anbarkeh *et al*.[17] Briefly, sections were incubated with primary antibody against laminin (ab11575) followed by secondary antibody (ab205718). The intensity of immunostaining was quantified using ImageJ Software. Optical density (OD) was calculated as follows:

$$OD = \log \frac{\text{Maximum intensity}}{\text{Mean intensity}}$$

Western blot. Western blot analysis was performed based on previously published protocols.[18,19] First, kidney tissues were homogenized in radioimmunoprecipitation assay buffer (with protease inhibitor cocktail and phenylmethylsulfonyl fluoride, Sigma-Aldrich). Then, the tissues were centrifuged (15,000 rpm) at 4.00 ˚C for 15 min. After separating the supernatant, the protein concentration in the samples was determined using the Bradford assay. Samples were mixed with 2 × sodium dodecyl sulfate sample buffer at a 1:1 ratio and heated at 95.00 ˚C for 10 min. Proteins were separated on a 12.00% sodium dodecyl sulfate –polyacrylamide gel and transferred onto polyvinylidene difluoride membranes (Bio-Rad Laboratories, Hercules, CA, USA). Membranes were blocked with 5.00% non-fat dry milk (or 5.00% bovine serum albumin (Sigma-Aldrich) for phosphorylated proteins) and then, incubated with primary antibodies against ERK1/2 (rabbit polyclonal, Cell Signaling Technology, Danvers, USA) and p-ERK1/2 (rabbit polyclonal, Cell Signaling Technology), followed by horseradish peroxidase-conjugated anti-rabbit immunoglobulin G secondary antibody (1:3,000; Cell Signaling Technology, Danvers, USA;). Finally, enhanced chemiluminescence was used to identify protein bands. The integrated ODs of bands were measured using Alliance 4.7 Gel Doc (Alliance 4.7; UVItec, Cambridge, UK). Protein bands were analyzed *via* UVtec Software (UVITEC, Cambridge, UK). The GAPDH (1:1,000; rabbit monoclonal antibody, Cell Signaling Technology) was used as an internal loading control.

Superoxide dismutase (SOD) activity analysis. The SOD activity was measured by Winterbourn protocol.[20] Briefly, 20.00 µL of tissue homogenate was added to a reaction mixture, containing ethylenediamine-tetraacetic acid, sodium cyanide, and nitroblue tetrazolium. The reaction was incubated at 37.00 ˚C for 5 - 8 min, and absorbance was measured at 560 nm. One unit of SOD activity was defined as the amount of enzyme required to inhibit the reduction of nitroblue tetrazolium by 50.00%.

Glutathione reductase (GR) activity analysis. The GR measurement was performed by Mohandas *et al.* protocol.[21] Briefly, GR activity was determined based on the rate of nicotinamide adenine dinucleotide phosphate (NADPH) oxidation to $NADP^+$, being monitored by measuring the decrease in absorbance at 340 nm, using a spectrophotometer at room temperature.

Glutathione S-transferase (GST) activity analysis. The GST enzyme activity was measured using Habig and Jakoby's method.[22] Briefly, the reaction mixture contained potassium phosphate buffer (50.00 mM; pH: 7.00), ethylenediaminetetraacetic acid (1.00 mM), reduced glutathione (GSH), and 1-chloro-2,4-dinitrobenzene as substrate. The reaction was initiated by adding tissue homogenate, and the increase in absorbance at 340 nm was monitored spectrophotometrically.

Glutathione peroxidase (GSH-Px) activity analysis. The GSH-Px was measured according to the method of Paglia and Valentine.[23] Briefly, GSH-Px catalyzes the reduction of peroxides by GSH, resulting in the formation of glutathione disulfide (GSSG). In the presence of GR and NADPH, GSSG is converted back to GSH, accompanied by oxidation of NADPH to $NADP^+$. The decrease in absorbance at 340 nm was monitored spectrophotometrically.

Statistical analysis. Data analysis was done by SPSS Software (version 16.0; SPSS, Inc., Chicago, USA). Normality of data distribution was assessed prior to analysis. One-way analysis of variance followed by Tukey's *post hoc* test was used. Data are presented as mean ± standard error of the mean, and $p < 0.05$ was considered statistically significant.

Results

The influence of DZN and BM on renal glomeruli. Representative photomicrographs and quantitative analyses are shown in Figure 1. The PAS and JMS staining methods showed a decrease in mesangial matrix accumulation and thinning of the glomerular basement membrane, respectively, in the DZN-treated rats compared to the control rats ($p < 0.05$; Figs. 1 and 2). Moreover, reduced laminin expression intensity in the DZN group compared to the control group was shown ($p < 0.05$). In rats treated with BM, mesangial matrix accumulation, glomerular basement membrane thickness, and laminin expression did not differ significantly from those of the control group ($p > 0.05$). Treatment with BM increased the mesangial matrix accumulation in the BM and DZN + BM groups, being significantly higher than that in the DZN group ($p < 0.05$). As seen in Figure 2, administration of BM notably increased the thickness of the glomerular basement membrane in the BM and DZN + BM groups compared to the DZN group ($p < 0.05$). Laminin expression in the renal glomeruli was significantly higher in the BM and DZN + BM groups than DZN group ($p < 0.01$ and $p < 0.05$, respectively).

The influence of DZN and BM on the expression of ERK and p-ERK proteins. Representative western blot images of ERK and p-ERK are shown in Figure 3A. Western blot analysis results showed that DZN administration significantly decreased p-ERK levels in the kidney compared to the control group ($p < 0.01$; Fig. 3B). Similarly, ERK protein levels were significantly reduced in the DZN-treated group compared to the control group ($p < 0.05$; Fig. 3C). Moreover, rats treated with BM were not significantly different regarding ERK and p-ERK levels compared to the control group ($p > 0.05$). The rats received 150 mg/kg BM showed a significant increase in p-ERK protein levels ($p < 0.001$; Fig. 3B) and ERK expression ($p < 0.01$; Fig. 3C).

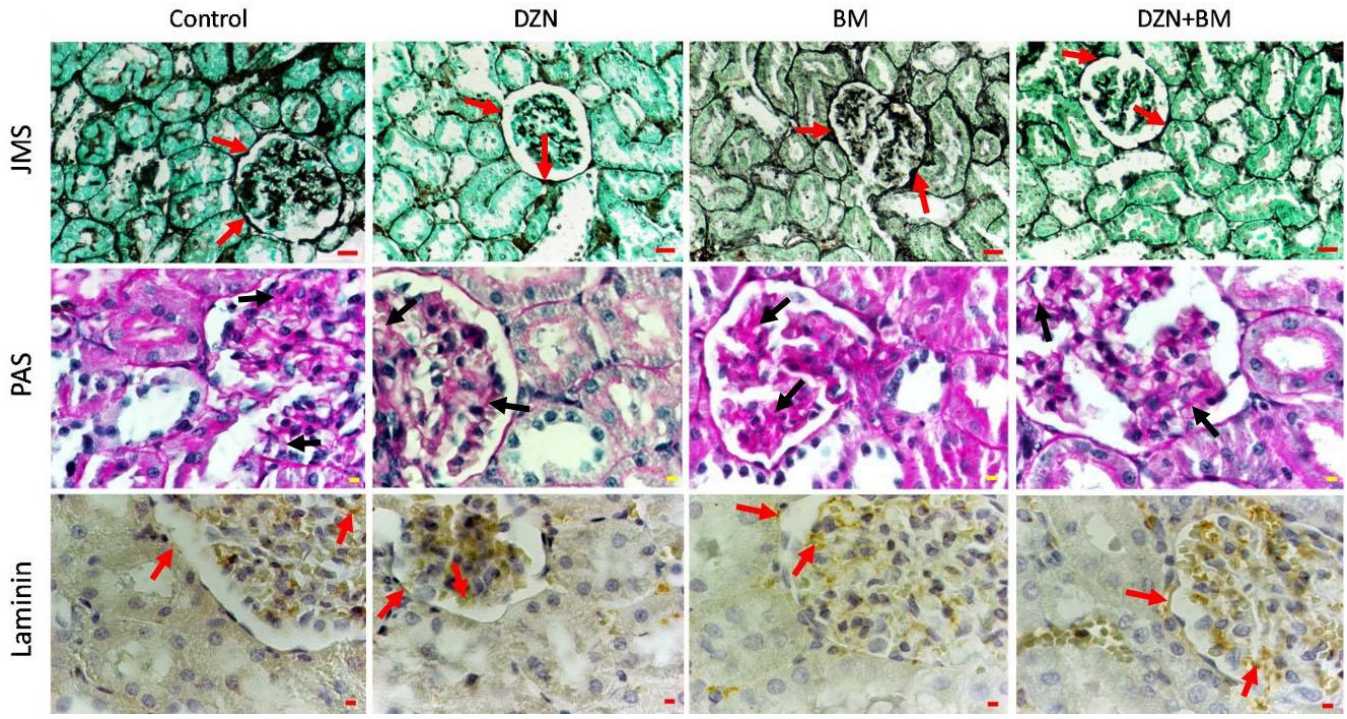


Fig. 1. Photomicrographs showing the effect of diazinon (DZN) and *Biebersteinia multifida* (BM) on renal glomeruli. Representative images of Jones methenamine silver (JMS), Periodic acid–Schiff (PAS), and laminin immunohistochemically -stained sections from control, DZN, BM, and DZN + BM groups are shown. In JMS-stained sections, arrows indicate thickening of the glomerular basement membrane, in PAS-stained sections, arrows indicate mesangial matrix accumulation, and in laminin immunohistochemically -stained sections, arrows indicate laminin expression along the basement membrane. Scale bars = 100 µm for PAS and laminin images and 40.00 µm for JMS images.

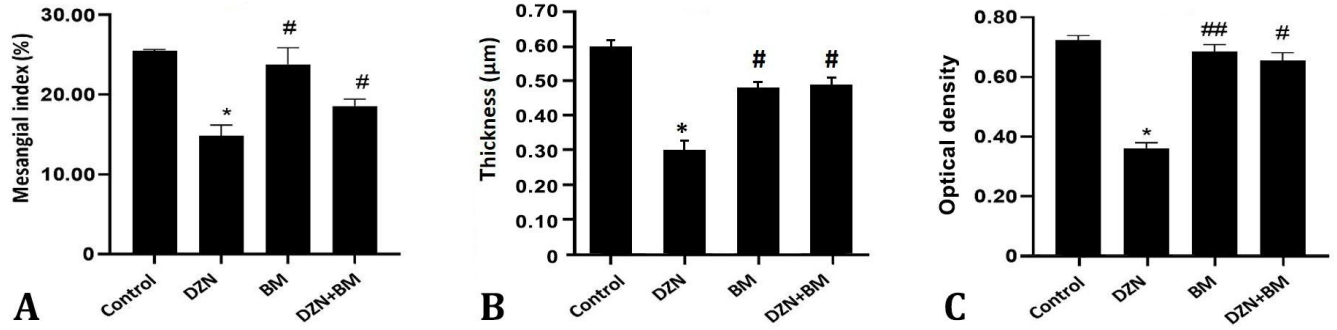


Fig. 2. Quantitative analysis includes **A)** Mesangial matrix index, **B)** Thickness of the glomerular basement membrane, and **C)** Laminin optical density in renal glomeruli. #$p < 0.05$ and ##$p < 0.01$ *versus* DZN group; *$p < 0.05$ *versus* control group.

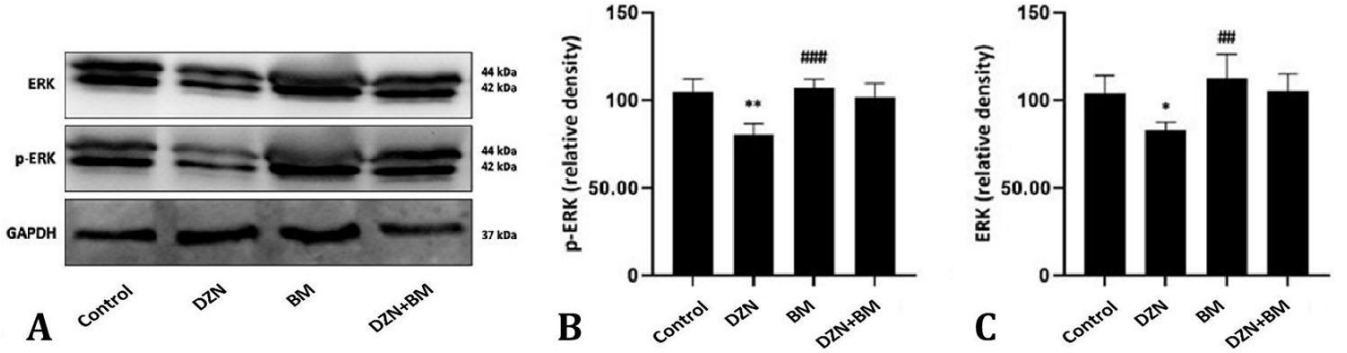


Fig. 3. The effect of diazinon (DZN) and *Biebersteinia multifida* (BM) extract on renal extracellular signal-regulated kinase (ERK) and phosphorylated (p)-ERK proteins expression. Representative western blot images are shown in panel A. Densitometric analyses are presented in panels B and C. Statistical significance is indicated as ## $p < 0.01$, and ### $p < 0.001$ *versus* DZN group; * $p < 0.05$ and ** $p < 0.01$ *versus* control group.

Protective effect of BM on the activities of SOD, GST, GSH-Px, and GR in kidney after DZN administration. Compared to the control group, renal GR and GSH-Px activities were markedly reduced following DZN administration, whereas GST and SOD activities were significantly elevated ($p < 0.05$). No significant difference in SOD, GST, GSH-Px, and GR activities was found between the control and BM-administered groups ($p > 0.05$). In the DZN + BM group, GR and GSH-Px activities were significantly increased compared to the DZN group ($p < 0.05$). In contrast, GST activity was significantly decreased in the DZN + BM group relative to the DZN group ($p < 0.05$). Although SOD activity was reduced in the DZN + BM group compared to the DZN group, this difference did not reach statistical significance ($p > 0.05$; Table 1).

Discussion

The present study evaluated the protective effects of the methanolic extract of BM against DZN-induced renal injury, with a particular focus on OS and ERK/p-ERK signaling. In the present study, DZN exposure significantly increased SOD and GST activities, while markedly decreasing GSH-Px and GR activities. This pattern is consistent with the biphasic response of the anti-oxidant defense system to organophosphate-induced OS.[24-27] The SOD and GST, rapidly-inducible components of the first-line anti-oxidant defense system, are transiently up-regulated as a compensatory mechanism against the initial burst of reactive oxygen species (ROS), a pattern also reported following DZN exposure in other rodent models.[28-30] In contrast, GSH-Px and GR, depending on the cellular GSH/NADPH pool, become functionally compromised under sustained pro-oxidant challenge, consistent with the marked reduction of both enzymes reported following DZN-induced hepatorenal OS.[26,31] Co-administration of BM restored GSH-Px and GR activities toward control values and attenuated the DZN-induced elevation of SOD and GST, suggesting that BM exerted its protective effect primarily by rebalancing the GSH-dependent arm of the anti-oxidant system rather than by uniformly suppressing all enzyme activities.[14,28] The observed protective effects of BM may be attributed, at least in part, to its rich phytochemical composition. Previous studies have identified various bio-active constituents in BM, including flavonoids, alkaloids, terpenoids, and essential oils.[11] Flavonoids are well known for their anti-oxidant properties and their ability to scavenge free radicals and modulate intracellular signaling pathways involved in cellular growth, differentiation, survival, and inflammation. Therefore, the restoration of anti-oxidant enzymes activities in the BM-treated groups may be linked to these bio-active compounds.[32,33] Notably, even though SOD activity exhibited partial restoration in the group receiving BM, the observed change failed to achieve statistical significance, implying that anti-oxidant enzymes display differential responsiveness to BM treatment in the context of DZN-induced OS. It is noteworthy that anti-oxidant enzymes activities following organophosphate exposure may either increase or decrease depending on factors, such as the dose administered, time point of measurement, and adaptive capacity of the organism or tissue involved.[34,35]

A key finding of the present study is the significant down-regulation of ERK and p-ERK expressions following DZN exposure. The MAPK/ERK pathway plays a crucial role in regulating cellular responses to stress, including proliferation, differentiation, and survival.[36] Previous studies have reported that organophosphates can modulate the MAPK signaling pathway through the induction of OS.[37] Oxidative stress is a key regulator of MAPK/ERK signaling in kidney injury. It can activate ERK through stress-related pathways, leading to downstream effects, such as inflammation and apoptosis, *via* mediators, including cyclooxygenase2, p53, Bax, and caspase-3. In addition, ROS-induced activation of MAPK pathways can promote inflammatory genes expression through Nuclear factor kappa B signaling, thereby contributing to the renal tissue damage.[38] In general, extracellular stimuli, including mitogens and genotoxic stress, activate ERK signaling.[39] In this context, the observed decrease in ERK and p-ERK expressions in the DZN-treated group may indicate an inability to mount adaptive survival signaling, rather than active suppression of injury pathways. This kind of impairment could heighten renal cells vulnerability to oxidative damage and hinder their capacity to preserve cellular homeostasis. Prior studies have described similar context-dependent functions of ERK signaling in promoting both cell survival and cell death, underscoring its dual role in response to cellular stress.[40,41] Nonetheless, the effects of ERK signaling are strongly context-dependent; when OS is severe or sustained, dysregulation of signaling pathways may occur, resulting in impaired ERK activity and reduced cellular adaptive capacity.[42]

Table 1. Effects of diazinon (DZN) and *Biebersteinia multifida* DC (BM) on superoxide dismutase (SOD), glutathione S-transferase (GST), glutathione peroxidase (GSH-Px), and glutathione reductase (GR) activity.

Parameters	Control	DZN	BM	DZN + BM
SOD activity (U *per* mg tissue)	1.40 ± 0.30	2.60 ± 0.40*	1.30 ± 0.30#	2.20 ± 0.50
GST activity (nmol *per* mg tissue)	4.70 ± 0.20	8.70 ± 0.50*	4.30 ± 0.30#	7.90 ± 0.30#
GSH-Px activity (nmol *per* mg tissue)	1.45 ± 0.06	0.52 ± 0.09*	1.49 ± 0.08#	1.36 ± 0.07#
GR activity (nmol *per* mg tissue)	1.49 ± 0.07	0.56 ± 0.11*	1.57 ± 0.11#	1.45 ± 0.06#

*$p < 0.05$ *versus* control group; #$p < 0.05$ *versus* DZN group.

The ECM and basement membrane are dynamic structures, composed of various molecules, including collagen, proteoglycans, glycoproteins, fibronectin, laminin, and associated regulatory proteins. These components play essential roles in regulating cell adhesion, migration, proliferation, and differentiation.[43]

The MAPK/ERK signaling plays a critical role in the synthesis and maintenance of ECM components, as well as kidney development, and disruption of this pathway has been associated with the progression of various renal disorders.[44] Therefore, the ERK down-regulation documented in this study may reflect compromised ECM maintenance and the resulting tissue damage.

Histological findings from the present study showed that DZN administration was associated with reduced laminin expression in renal corpuscles, suggesting impair-ment of basement membrane integrity. In contrast, increased laminin expression was observed in the DZN + BM and BM groups compared to the DZN group, indicating a potential protective effect of BM on basement membrane structure.

Previous studies investigating DZN-induced renal toxicity, as well as protective agents, such as alpha-lipoic acid, have also reported alterations in key ECM components, including fibronectin, laminin, and collagen, further supporting the involvement of ECM disruption in pesticide-induced renal injury.[17,26,29]

Further supporting these findings, Pizzurro *et al*. reported that DZN reduced fibronectin expression in astrocytes through OS-related mechanisms, while anti-oxidants, such as melatonin and N-tert-butyl-α-phenylnitrone, attenuate this effect.[45]

Treatment with BM extract significantly attenuated the effects of DZN, as evidenced by the restoration of anti-oxidant enzymes activities, increased ERK expression, and improvement of ECM integrity. These findings are consistent with our previous study, in which BM ameliorated DZN-induced OS and histopathological alterations in the hippocampus and improved anti-oxidant defense parameters.[14]

The protective effects observed in the present study may be attributed, at least in part, to the presence of bio-active compounds, such as flavonoids and other phenolic constituents in BM, possessing potent anti-oxidant activity,[9] and being capable of modulating intracellular signaling pathways. Flavonoids have been widely reported to scavenge ROS and regulate redox-sensitive signaling cascades, including MAPK pathways, thereby protecting cells against oxidative damage.[9,46,47]

Collectively, these findings support the hypothesis that DZN-induced OS contributes to the dysregulation of ERK signaling, which may subsequently impair ECM homeostasis during renal injury. The restoration of ERK expression following BM treatment suggests that modulation of redox-sensitive signaling pathways may represent one of the mechanisms underlying its renoprotective effects. However, further studies are warranted to elucidate the precise molecular mechanisms linking OS, ERK signaling, and ECM remodeling.

The DZN exposure may be associated with OS-induced alterations in renal anti-oxidant defense systems, leading to decreased expression of ERK and p-ERK proteins, impaired production of ECM components, particularly laminin, and consequent renal injury. In contrast, BM may attenuate DZN-induced renal damage, likely through its anti-oxidant constituents, thereby contributing to the preservation of renal structure and function.

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Conflict of interest

The authors have no conflict of interest.

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