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Harmaline improves diabetes-associated renal alterations in nicotinamide/streptozotocin-induced diabetic mice: Possible involvement of oxidative stress, inflammation, and renin–angiotensin system signaling

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ABSTRACT

Objective(s): Diabetic nephropathy (DN) is marked by structural and functional kidney damage, driven by oxidative stress, inflammation, and renin-angiotensin system (RAS) dysregulation. Harmaline (HAR), a β-carboline alkaloid with potent antioxidant and anti-inflammatory properties, is a promising multi-target therapeutic candidate.

Materials and Methods: Twenty-four male mice were randomized into vehicle control (CO), diabetic (DI; nicotinamide 120 mg/kg+streptozotocin 65 mg/kg), diabetic treated with HAR (DI/HAR; 30 mg/kg), or diabetic treated with metformin (DI/MET; 150 mg/kg). After ten days, renal tissues and blood were analyzed for oxidative stress, inflammatory and RAS markers, microRNA expression, and histopathological alterations.

Results: Harmaline reversed the changes in total oxidant status and malondialdehyde levels in kidney tissue of diabetic mice. Histopathological analysis showed significant improvement in kidney injury in diabetic mice receiving harmaline. Harmaline treatment decreased the expression of miR-125b, nuclear factor kappa B (NF-κB), tumor necrosis factor alpha (TNF-α), interleukin-1 beta (IL-1β), and interleukin-6 (IL-6). Also, in the antioxidant pathway, there was an increase in the levels of miR-200a, nuclear factor erythroid-related2 (Nrf2), and NAD(P)H quinone dehydrogenase 1 (Nqo1), and a decrease in ECH-like-1elate-associated protein1 (Keap1) in kidney tissue. This treatment also decreased the expression of angiotensin-converting enzyme 1 (ACE1) and angiotensin II receptor 1 (AT1R), as well as angiotensin II levels.

Conclusion: Harmaline confers renoprotection in experimental DN by simultaneously regulating oxidative stress, inflammatory signaling, and RAS pathways, supporting its potential as a multi-target therapeutic strategy. This study highlights HAR as a promising candidate for translational intervention in diabetic kidney disease.

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Introduction

Diabetic nephropathy (DN) is a serious complication of diabetes mellitus and a leading cause of end-stage renal disease worldwide, imposing substantial morbidity and mortality despite current therapeutic strategies. Clinically, DN is characterized by progressive albuminuria and a decline in glomerular filtration, ultimately leading to irreversible renal dysfunction (1).

Accumulating evidence indicates that oxidative stress and inflammation are central drivers of DN initiation and progression. These two processes alter the communication pathways between kidney cells, thereby increasing kidney damage and the advancement of DN (2). Therefore, therapeutic strategies targeting oxidative stress and inflammation have been proposed to attenuate DN progression (3). Nuclear factor erythroid-related factor 2, commonly referred to as Nrf2, is important in regulating the body's anti-oxidant mechanisms and maintaining the balance between oxidative and reductive processes within cells, making it a significant focus for preventing and managing diseases linked to oxidative stress (4). Kelch-like ECH-associated protein 1, also known as KEAP1, is responsible for regulating and controlling the function of Nrf2. Normally, KEAP1 degrades Nrf2 through ubiquitination and proteasomal degradation, thereby maintaining its levels at a low level. However, oxidative stress disrupts this interaction, allowing Nrf2 stabilization

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and nuclear translocation. Impairment of the Nrf2/KEAP1 axis has been implicated in the progression of diabetic renal injury (5-8). MicroRNAs (miRs) are small RNA molecules that help control gene expression but do not make proteins. Among regulatory microRNAs, miR-200a has been shown to enhance Nrf2 signaling by suppressing KEAP1 expression (9, 10).

Inflammatory signaling further amplifies renal damage in DN. Activation of nuclear factor kappa B (NF-κB) under diabetic conditions enhances the transcription of pro-inflammatory cytokines, including tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), and interleukin-1β (IL-1β), thereby perpetuating renal inflammation and fibrosis (11, 12). Dysregulation of the renin–angiotensin system (RAS) represents a pivotal contributor to diabetic renal injury and remains a major therapeutic target (13). Overactivation of the RAS, particularly through angiotensin II (Ang II), contributes to oxidative stress, inflammatory signaling, cellular proliferation, and proteinuria in diabetic kidneys. These interconnected pathways suggest that multi-target modulation of oxidative stress, inflammation, and RAS activation may offer superior therapeutic benefit (14). Importantly, these pathways are mechanistically interconnected, as Ang II-driven oxidative stress can activate inflammatory signaling pathways, including NF-κB, thereby promoting renal inflammation and fibrosis (15).

Natural bioactive compounds with anti-oxidant and anti-inflammatory properties have gained increasing attention as potential adjunctive therapies in DN. Harmaline (HAR), a β-carboline alkaloid derived from *Peganum harmala* and *Banisteriopsis caapi* (16), has demonstrated potent anti-oxidant and anti-inflammatory activities in experimental models, partly through modulation of redox-sensitive signaling pathways (17, 18). However, whether Harmaline can confer renoprotection through coordinated modulation of the Nrf2/KEAP1 axis, NF-κB signaling, and RAS activation in DN has not yet been comprehensively investigated. Therefore, the present study was designed to investigate the renoprotective effects of Harmaline in nicotinamide/streptozotocin (NA/STZ)-induced diabetic mice. We hypothesized that Harmaline attenuates diabetic renal injury by activating Nrf2-mediated anti-oxidant defenses and suppressing NF-κB-driven inflammatory responses and RAS signaling. Elucidating these mechanisms may provide mechanistic insight into the development of multi-target, pathway-oriented therapeutic strategies for DN.

Materials and Methods

Chemical materials

Nicotinamide, streptozotocin, Harmaline, metformin (MET), and Bradford reagent were purchased from Sigma-Aldrich, a company based in the United States. Sodium citrate dihydrate and citric acid were obtained from Merck (Germany). Ketamine and xylazine were purchased from Alfasan, Netherlands.

Animals and experimental design

Six- to eight-week-old male NMRI mice were received from the Animal Center of Ahvaz Jundishapur University of Medical Sciences, and used to study the protective effect of Harmaline against diabetic kidney disease. All experimental animals in the laboratory at the Animal Ahvaz Jundishapur University of Medical Sciences were maintained under specific conditions. All mice were maintained at 22 °C and a 12-hr light-dark cycle, and had unlimited access to standard food and water. This study was conducted in accordance with the National Institutes of Health guidelines for the use of laboratory animals and approved by the Ethics Committee of Ahvaz Jundishapur University of Medical Sciences (IR.AJUMS.AEC.1403.012). Mice were randomly divided into: (1) a control (CO) group (n=6), (2) a diabetic (DI) group (n=6), (3) a diabetic group treated with Harmaline (n=6), and (4) a diabetic treated with metformin (DI/MET) group (n=6). After 12 hr of fasting, the type 2 diabetes model was induced with NA at a dose of 120 mg/kg body weight, dissolved in normal saline, and injected into the animals. 15 min after nicotinamide administration, STZ was injected at a dose of 65 mg/kg (dissolved in freshly prepared citrate buffer with pH 4.5) (19). 7 days after NA and STZ injection, fasting blood glucose (FBG) levels were measured using blood samples collected from the tail vein. Mice with FBG levels above 200 mg/dl were selected to confirm induced diabetes. These diabetic mice were then given Harmaline (30 mg/kg)(20), metformin (150 mg/kg)(21), or the appropriate solvent by gavage for 10 days. Harmaline was dissolved in normal saline. The control mice and the diabetic control received an equivalent volume of normal saline, which served as the vehicle for the test compound.

On the last day of the experiment, to measure renal factors, 24-hr urine samples were collected from animals in a metabolic cage (20 × 20 × 15 cm) one day before the end of the treatment period. After the blood glucose level was measured again, the mice were injected with a ketamine-xylazine combination to induce unconsciousness. Blood samples were taken using cardiac puncture. The blood was spun at 3000 rpm for 10 min to separate the plasma, which was then stored at -20 degrees Celsius for testing. Next, kidney tissue from the mice was removed for further investigation.

Biochemical analysis

Sodium, potassium, creatinine, and albumin in plasma and urine samples were measured using an autoanalyzer (model BT3000, manufactured in Italy) and Biorexfars kits from Iran. In addition, blood urea nitrogen (BUN), urinary urea, and glucose levels were analyzed in this manner.

Renal function and fibrosis-related marker analysis

To assess kidney function, the study analyzed the sodium excretion fraction (FENa+), potassium excretion fraction (FEK+), 24-hr urine albumin, the urinary albumin-to-creatinine ratio (UACR), and urinary excretion rates of sodium, potassium, urea, and creatinine, and the concentration of TGF-β, a renal fibrosis-associated marker.

Evaluation of oxidative stress and anti-oxidants in the kidney

Initially, kidney tissue was combined with cold phosphate-buffered saline solution at pH 7.4 and incorporating protease inhibitors. Then, it was centrifuged for 20 min at 4000 rpm at 4 °C. The clear liquid that rose to the top, called the supernatant, was used to measure the oxidant status, including malondialdehyde (MDA) content and total anti-oxidant capacity (TAC), using colorimetric methods and standard assay kits from Zell Bio GmbH, Germany. The supernatant sample and the Bradford method were used to assess protein concentration.

Real-Time PCR analysis

Total RNA was extracted from kidney tissues according to the manufacturer's instructions for the Parstous RNA extraction kit Tehran, Iran. Complementary DNA (cDNA) was synthesized from the extracted RNA using the QuantiNova™ Reverse Transcription Kit (Parstous, Tehran,

Table 1. Sequences of the gene-specific primers used for real-time PCR

	Forward (5′ -3′)	Reverse (5′ -3′)
GAPDH	CATCACTGCCACCCAGAAGACTG	ATGCCAGTGAGCTTCCCGTTCAG
ACE1	TCAGCGGAATGGCGAAGTCCTA	CGGTCATACTCTTCCACGAACC
AT1R	GCCATTGTCCACCCGATGAAGT	ACACATTTCGGTGGATGACGGC
Keap-1	ATCCAGAGAGGAATGAGTGGCG	TCAACTGGTCCTGCCCATCGTA
Nqo1	GCCGAACACAAGAAGCTGGAAG	GGCAAATCCTGCTACGAGCACT
U6	GCTCGCTTCGGCAGCACA	-
miR-200a	TCTTACCGGACAGTGCTG	-
miR-125b	CCCTGAGACCCTAACTTG	-

ACE1: Angiotensin-converting enzyme 1; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase; AT1R: Angiotensin II receptor 1; Keap-1: Kelch-like ECH-associated protein 1

Iran) following the recommended protocol. To isolate microRNA, the FavorPrepTM miRNA Isolation Kit (South Korea) was employed. MicroRNA reverse transcription was performed using the cDNA Synthesis kit (Parstous kit, Iran). The mRNA and microRNA expression levels were determined using the 2X SYBR Green Real-Time PCR kit produced by Pars Tous, Iran, and the Lava96 Real-Time PCR Detection System (DaAnGene Co Ltd) for real-time PCR. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) served as the internal control to adjust for variations in mRNA levels, whereas U6 was used as the internal control for microRNA measurements. The relative changes in mRNA and microRNA expression between groups were calculated using the ΔΔCt method. The primers specifically used in this study are detailed in Table 1.

Nrf2 and NF-kB p65 analysis by Western blot

Kidney tissues were first homogenized, and then protein concentration was determined using the Bradford method. Proteins were then electrophoresed using SDS-PAGE and subsequently transferred to nitrocellulose membranes. The membranes were then incubated with primary and secondary antibodies specific for Nrf2 (Santa Cruz Biotechnology, USA) and NF-κB p65 (Santa Cruz Biotechnology, USA).

Measurement of cytokine levels

In kidney homogenate samples, the levels of transforming growth factor beta (TGF-β), TNF-α, IL-1β, and IL-6 were measured by enzyme-linked immunosorbent assay (ELISA) using kits from SunLong Biotech, China. The level of Ang II was measured in the same way, but using an LS Bio kit from the United States.

Histopathological analysis

Kidney tissue was stored in 10% formalin to prevent structural changes. After that, the tissue went through alcohol treatment to remove any water. Then, it was placed in paraffin so that thin slices could be made. These slices were cut to a thickness of 4-6 micrometers and stained with hematoxylin and eosin (H&E) to enhance the visibility of cellular structures under a microscope. The histological changes, pyknosis, Glomerular disorganization, Congestion, and Epithelial sloughing were examined. For every animal, six slides were made. The degree of damage was determined by examining a random area and calculating the kidney's affected area. Kidney damage was assessed using a scoring system:

1. If the kidney tissue was normal, a score of 0 was assigned.
2. If the kidney tissue damage was mild, a score of 1 was assigned.
3. If the kidney tissue damage was moderate, a score of 2 was assigned.
4. If the kidney tissue damage was severe, a score of 3 was assigned.

Statistical analysis

Data are presented as mean ± SEM (standard error of the mean). For data that followed a normal distribution, the means between different groups were compared using one-way analysis of variance with Tukey's *post hoc* test. When the data did not conform to a normal distribution, the Kruskal-Wallis and Dunn's tests were used. All statistical analyses were managed using SPSS version 25, with graphics generated in GraphPad Prism version 9. A *P*-value below 0.05 was taken to indicate statistical significance.

Results

Harmaline helped improve fasting blood glucose levels and various chemical levels in the blood and urine

In this study, the effects of diabetes induction and Harmaline treatment on serum and 24-hr urinary biochemical parameters were evaluated using metabolic cages. Type 2 diabetes was induced by NA/STZ administration. Diabetic mice showed a significant increase in fasting blood glucose (FBG) levels compared with the control group (Figure 1A; $P<0.001$). In addition, increased urinary glucose levels and urine volume were observed in diabetic mice (Figure 2A and Figure 2G; $P<0.001$). Treatment with Harmaline and metformin significantly reduced these parameters compared with the untreated diabetic group ($P<0.001$ for FBG and urinary glucose levels, $P<0.01$ for urine volume). Serum sodium levels were significantly decreased in diabetic mice compared with the control group (Figure 1B; $P<0.001$), whereas urinary sodium levels were increased (Figure 2B; $P<0.001$). Administration of Harmaline and metformin significantly restored serum sodium levels ($P<0.01$) and reduced urinary sodium levels ($P<0.001$) compared with the diabetic group. Diabetic mice exhibited significantly higher serum and urinary potassium levels compared with the control group (Figures 1C and 2C; $P<0.001$). Treatment with Harmaline and metformin significantly decreased potassium levels compared with the diabetic group ($P<0.001$). Moreover, the reduction in serum potassium levels was more pronounced in the Harmaline-treated group compared with the metformin-treated group ($P<0.05$). Serum biochemical analysis revealed a significant decrease in albumin levels ($P<0.001$) and an increase in creatinine ($P<0.01$) and blood urea nitrogen (BUN) levels

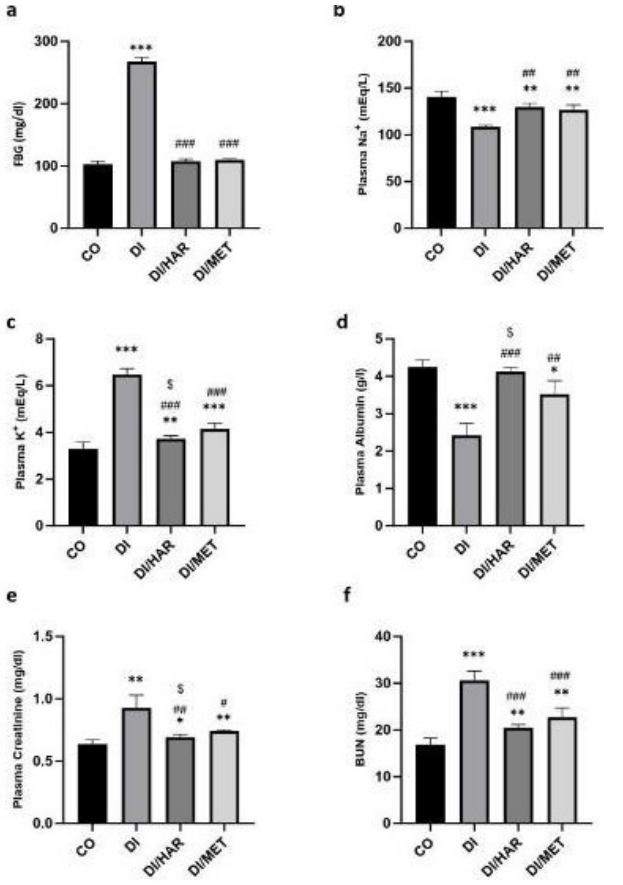

Figure 1. Effects of HAR on:
a) Mouse fasting blood glucose, b) Plasma sodium, c) Plasma potassium, d) Plasma albumin, e) Plasma creatinine, and f) BUN
CO group, DI control group, DI treated with HAR, and DI treated with MET (positive control group).
Data are presented as mean±SEM (n= 6/group). Statistical analysis was performed using one-way ANOVA followed by Tukey's *post hoc* test. * Compared to CO and # compared to DI. $ indicates comparison between DI/HAR and DI/MET (positive control) groups. Symbols 1, 2, and 3 indicate statistically significant differences with $P<0.05$, $P<0.01$, and $P<0.001$, respectively
HAR: Harmaline; BUN: Blood urea nitrogen; CO: Control; DI: Diabetic; MET: Metformin

($P<0.001$) in diabetic mice compared with control animals (Figure 1D–F). Treatment with Harmaline partially restored albumin levels ($P<0.001$) and increased creatinine ($P<0.01$) and BUN levels ($P<0.001$). Treatment with metformin increased albumin levels ($P<0.01$) and decreased creatinine ($P<0.05$) and BUN levels ($P<0.001$). Harmaline treatment resulted in a greater increase in serum albumin levels and a greater reduction in serum creatinine levels compared with metformin treatment ($P<0.05$). Analysis of 24-hr urine samples showed increased urinary albumin and urea levels and decreased urinary creatinine levels in diabetic mice compared with the control group (Figure 2D–F; $P<0.001$). Treatment with Harmaline and metformin significantly improved these urinary parameters compared with untreated diabetic mice ($P<0.001$ and $P<0.01$).

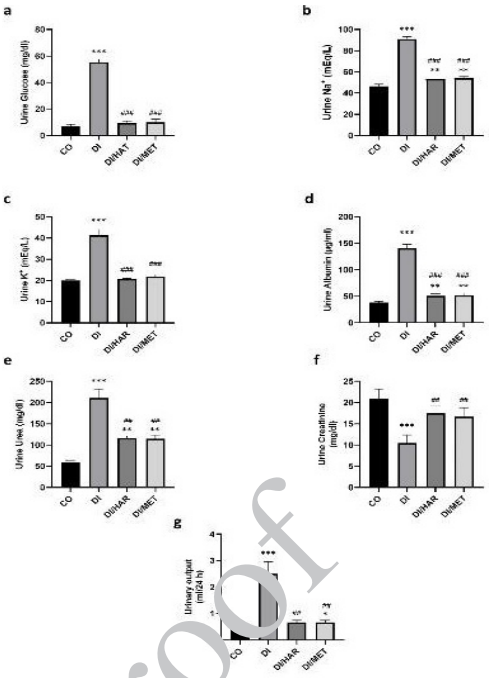

Figure 2. Effects of HAR on:
a) Mouse urine glucose, b) Urine sodium, c) Urine potassium, d) Urine albumin, e) Urine urea, f) Urine creatinine, and g) Urine volume
CO group, DI control group, DI treated with HAR, and DI treated with MET (positive control group).
Data are presented as mean±SEM (n= 6/group). Statistical analysis was performed using one-way ANOVA followed by Tukey's *post hoc* test. * Compared to CO and # compared to DI. Symbols 1, 2, and 3 indicate statistically significant differences with $P<0.05$, $P<0.01$, and $P<0.001$, respectively
HAR: Harmaline; CO: Control; DI: Diabetic; MET: Metformin

Harmaline improves urinary excretion rate of various substances

The diabetic group had significantly higher urinary excretion rates of sodium, potassium, creatinine, and urea compared with the control group ($P<0.001$). Compared with the diabetic group, both the Harmaline and metformin

Table 2. Effects of HAR on UER of sodium, potassium, albumin, urea, and creatinine in mice

Parameters	CO	DI	DI/HAR	DI/MET
UER of Sodium (mEq/day)	25.53±1.02	227.83±17.55***	35.45±1.81###	36.03±1.98###
UER of Potassium (mEq/day)	11.05±0.54	103.60±8.07***	13.80±0.67###	14.49±0.70###
UER of Urea (mg/day)	31.83±1.83	526.75±37.90***	77.7±4.65###	76.65±4.43###
UER of Creatinine (mg/day)	11.38±0.44	26.58±3.08***	11.70±0.85###	11.11±0.83###

Data are represented as mean±SEM (n=6). * Compared to CO and # compared to DI. 1, 2, and 3 symbols represent statistically significant differences with $P<0.05$, $P<0.01$, and $P<0.001$, respectively
HAR: Harmaline; UER: Urinary excretion rate; CO: Control group, DI: Diabetic control group, UER: Urinary excretion rate

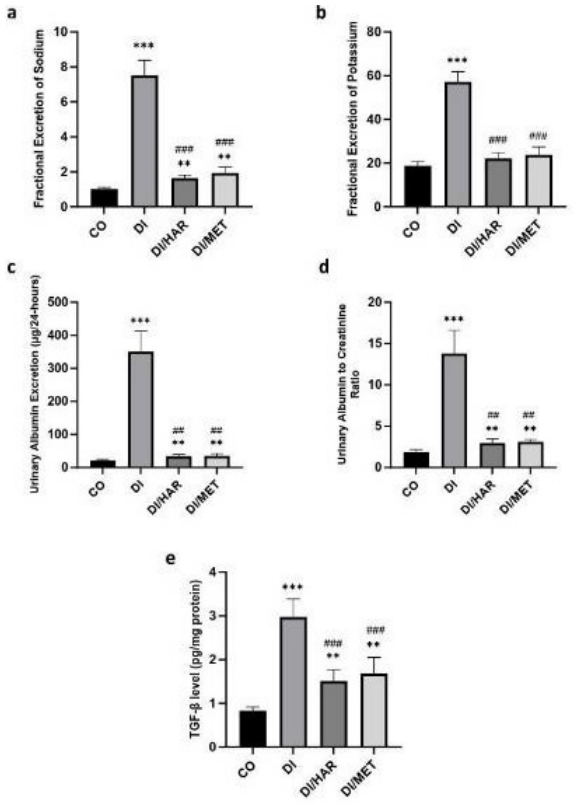

Figure 3. Effects of HAR on:
a) Mouse fractional excretion of sodium, b) Fractional excretion of potassium, c) 24 hr urinary albumin excretion, d) urinary albumin-to-creatinine ratio, e) TGF-β levels in kidney tissue.
CO group, DI control group, DI treated with HAR, and DI treated with MET (positive control group).
Data are presented as mean±SEM (n=6/group). Statistical analysis was performed using one-way ANOVA followed by Tukey's *post hoc* test. * Compared to CO and # compared to DI. 1, 2, and 3 symbols represent statistically significant differences with $P<0.05$, $P<0.01$, and $P<0.001$, respectively
HAR: Harmaline; TGF-β: Transforming growth factor beta; CO: Control; DI: Diabetic; MET: Metformin

groups showed a significant decrease in urinary excretion rates of sodium, potassium, creatinine, and urea ($P<0.001$). No statistically significant differences were detected between the treatment and positive control groups (Table 2).

Harmaline and metformin help in treating type 2 nephropathy

In this study, FENa+ and FEK+ were examined to evaluate renal function further. As illustrated in Figures 3a and 3b, the levels of FENa+ and FEK+ were notably greater in the group with diabetes ($P<0.001$). Diabetic mice treated with Harmaline and metformin returned FENa+ and FEK+ to their reduced levels ($P<0.001$). As shown in Figures 3c and 3d, two important indicators of DN, namely the amount of albumin excreted in urine over 24 hr and UACR, were significantly higher in diabetic mice ($P<0.001$). The diabetic group receiving Harmaline and metformin restored these alterations ($P<0.01$). In the study of kidney fibrosis, levels of one cytokine called TGF-β were assessed. As expected, the study findings in Figure 3e showed that TGF-β levels were significantly increased in diabetic mice ($P<0.001$). The diabetic group receiving Harmaline and metformin showed a significant reduction in this cytokine level ($P<0.001$). In this study, the pathology and histopathology of kidney tissue were analyzed, and treatment using Harmaline

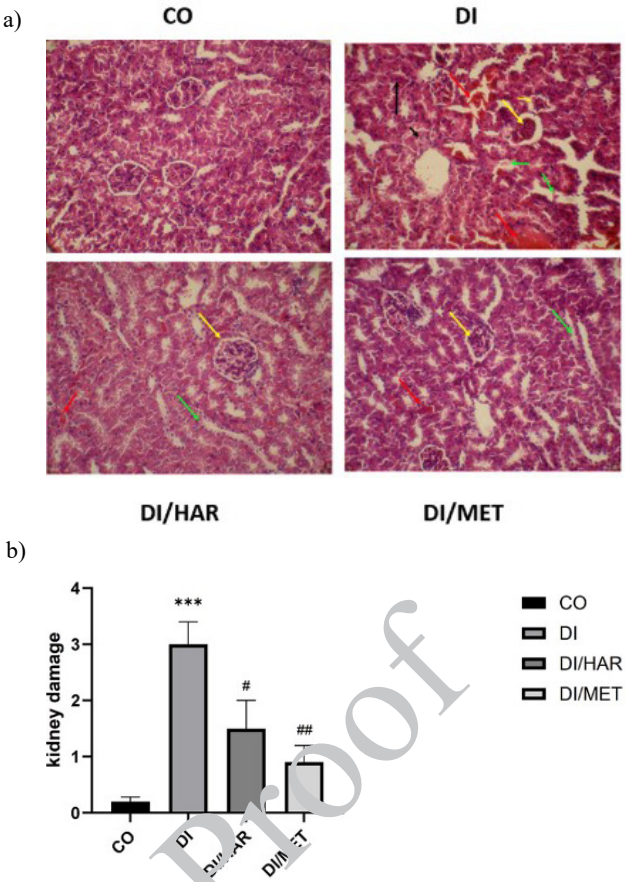

Figure 4. a) Effects of HAR on histology of kidney tissue in NA & STZ, STZ-induced type 2 diabetic mice: black, yellow, red, and green arrows indicate PK, Gd, Co, and Es, respectively. b) Kidney damage
CO group, DI control group, DI treated with HAR, and DI treated with MET (positive control group).
Data are presented as mean±SEM (n= 4/group). Statistical analysis was performed using one-way ANOVA followed by Tukey's *post hoc* test. * Compared to CO and # compared to DI. 1, 2, and 3 symbols represent statistically significant differences with $P<0.05$, $P<0.01$, and $P<0.001$, respectively. Representative samples are shown with H&E staining (Magnification × 200)
ES: Epithelial sloughing; Co: Congestion; Gd: Glomerular disorganization; PK: Pyknosis NA: Nicotinamide; STZ: Streptozotocin; HAR: Harmaline; CO: Control group, DI: Diabetic control group, MET:Metformin

($P<0.05$) and metformin ($P<0.01$) resulted in better kidney structure compared to the group with diabetes, as illustrated in Figures 4a and 4b.

Harmaline affects the intrinsic renal RAS, which is important in kidney disease

Given the connection between RAS and oxidative stress and inflammation, the relationship between local RAS in the kidney, as well as its components, was investigated. In this study, parts of the RAS were examined, and how Harmaline and metformin affect them. The findings from the RAS system assessment are presented in Figure 5(a and b), demonstrating a significant rise in Angiotensin-converting enzyme 1 (ACE1) mRNA levels in diabetic mice, as well as a similar increase in angiotensin II receptor 1 (AT1R) mRNA levels in this group ($P<0.001$). Diabetic mice that received Harmaline and metformin had reduced levels of harmful ACE1 and AT1R mRNA ($P<0.01$). As illustrated in Figure 5C, the levels of angiotensin II were markedly elevated in mice with diabetes ($P<0.001$). In the Harmaline and metformin groups, angiotensin II levels returned to about normal ($P<0.001$). These results indicate activation of the

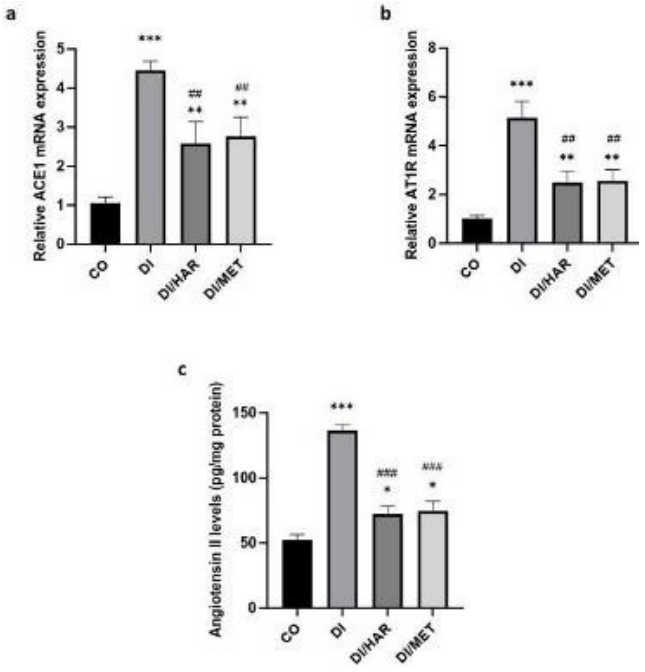

Figure 5. Effects of HAR on:
a) Mouse relative mRNA expression of (ACE1), b) Relative mRNA expression of ATR1, and c) Angiotensin II levels in the mouse kidney tissue
CO group, DI control group, DI treated with HAR, and DI treated with MET(positive control group).
Data are presented as mean±SEM (n=6/group). Statistical analysis was performed using one-way ANOVA followed by Tukey's *post hoc* test. * Compared to CO and # compared to DI. Symbols 1, 2, and 3 indicate statistically significant differences with $P<0.05$, $P<0.01$, and $P<0.001$, respectively
HAR: Harmaline; ATR1: Angiotensin-converting enzyme 1; CO: Control; DI: Diabetic; MET: Metformin

RAS system in type 2 diabetes.

Harmaline reduced inflammation with effects on miR-125b, NF-κB p65, TNF-α, IL-1β, and IL-6.

The strong link between inflammation and kidney problems in people with diabetes and the role of microRNAs in the body encouraged us to investigate the role of inflammation and the microRNAs that affect it. As shown in Figures 6a and 6b, the diabetic group exhibited higher levels of miR-125b ($P<0.001$), which was associated with a notable increase in the active form of NF-kB, specifically phosphorylated p65 NF-kB ($P<0.001$). Our results show that after treatment with harmaline and metformin, diabetic mice had lower levels of miR-125b compared to untreated diabetic mice ($P<0.001$). Similarly, in the groups treated with Harmaline ($P<0.01$) and metformin ($P<0.05$), the expression level of phosphorylated NF-κB p65 decreased, and this decrease was more pronounced ($P<0.01$) in the group receiving Harmaline (Figure 6b). In examining cytokine levels, including TNF-α, IL-1β, and IL-6, shown in Figure 6c, TNF-α levels in diabetic mice were increased compared to nondiabetic mice ($P<0.001$). Administration of Harmaline and metformin reduced this level ($P<0.01$), and the reduction was more significant in the group receiving Harmaline ($P<0.01$). As shown in Figure 6d, diabetic mice showed increased levels of IL-1β ($P<0.001$). Treatment with Harmaline and metformin significantly lowered these levels ($P<0.01$). Furthermore, in Figure 6E, Harmaline and metformin treatment significantly reduced interleukin-6 levels compared to diabetic mice ($P<0.001$).

Harmaline reduces oxidative stress with effects on miR-200a, Keap1, Nrf2, and Nqo1

In our study, which examined the levels of miR-200a, Keap1, and Nrf2 in the kidneys, diabetic mice showed higher miR-200a levels than control mice ($P<0.01$; Figure 7a). The harmaline- and metformin-treated groups showed increased levels of miR-200a ($P<0.05$) compared to the diabetic group. In Figure 7b, as expected, diabetic mice also had lower levels of Keap1 compared to control mice ($P<0.01$), and it was further reduced after harmaline and metformin treatment compared to diabetic mice ($P<0.01$). In diabetic mice, Nrf2 levels were higher ($P<0.05$) compared to the control group and showed a significant increase compared to the diabetic group after treatment with harmaline ($P<0.001$) and metformin ($P<0.01$). This increase in levels was greater in the harmaline group ($P<0.05$; Figure 7c). The level of NAD(P)H quinone dehydrogenase 1 (Nqo1), which is a target of Nrf2, was also significantly increased in diabetic mice ($P<0.05$). As shown in Figure 7d, treatment in both the harmaline ($P<0.01$) and metformin ($P<0.05$)

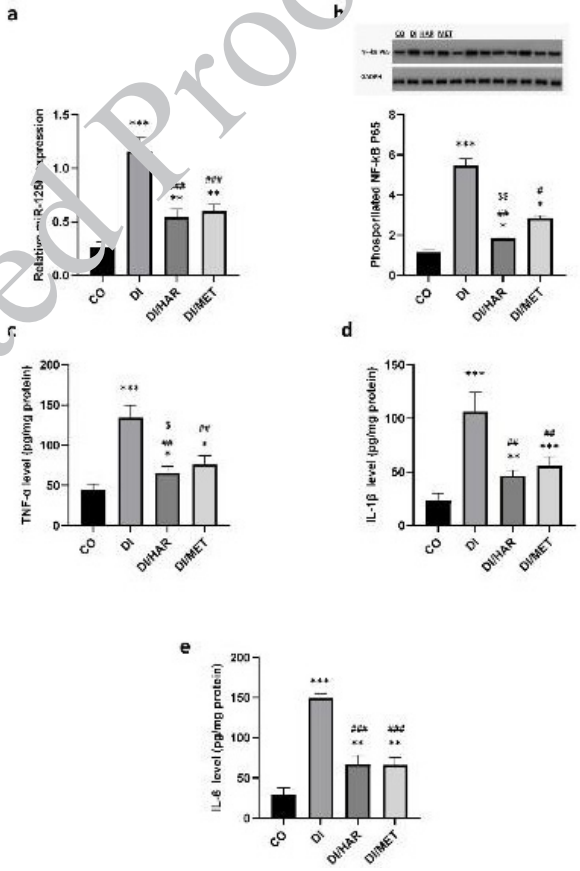

Figure 6. Effects of HAR on:
a) Mouse kidney tissue relative miR-125b expression, b) Kidney tissue relative NFkB-P65 (Western blot analysis), c) Kidney tissue TNF-α levels, d) Kidney tissue relative IL-1β levels, e) Kidney tissue IL-6 levels
CO group, DI control group, DI treated with HAR, and DI treated with MET (positive control group).
Data are presented as mean±SEM (n=6/group; for NFkB-P65 Western blot analysis, n=3). The lanes in the Western Bolt, from left to right, represent the NC, DC, HAR, and MET groups, respectively. Statistical analysis was performed using one-way ANOVA followed by Tukey's *post hoc* test. * Compared to CO and # compared to DI. $ indicates comparison between DI/HAR and DI/MET (positive control) groups. 1, 2, and 3 symbols represent statistically significant differences with $P<0.05$, $P<0.01$, and $P<0.001$, respectively
HAR: Harmaline; ATR1: Angiotensin-converting enzyme 1; CO: Control; DI: Diabetic; MET: Metformin; NF-κB: Nuclear factor kappaB; TNF-α: tumor necrosis factor alpha; IL-1β: Interleukin-1β; IL-6: Interleukin-6

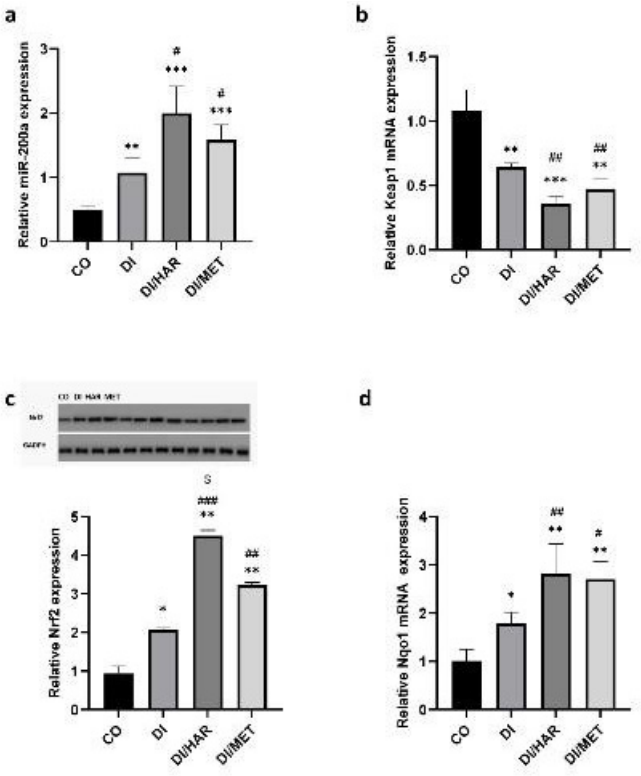

Figure 7. Effects of HAR on:
a) Mouse miR-200a, b) Keap1, c) Nrf2 (Western blot analysis), and d) Nqo1 relative expression in mouse kidney tissue
CO group, DI control group, DI treated with HAR, and DI treated with MET (positive control group).
Data are presented as mean±SEM (n=6/group; for Nrf2 Western blot analysis, n=3). The lanes in the Western Bolt, from left to right, represent the groups of NC, DC, HAR, and MET, respectively. Statistical analysis was performed using one-way ANOVA followed by Tukey's *post hoc* test. * compared to CO and # compared to DI. $ indicates comparison between DI/HAR and DI/MET (positive control) groups. 1, 2, and 3 symbols represent statistically significant differences with $P<0.05$, $P<0.01$, and $P<0.001$, respectively
HAR: Harmaline; CO: Control; DI: Diabetic; MET: Metformin; miR: Micro; Keap1: Kelch-like ECH-associated protein 1; Nrf2: Nuclear factor erythroid-related factor 2; Nqo1: Quinone dehydrogenase 1

groups resulted in a more significant increase compared to the diabetic groups. The higher levels of Nqo1 indicate that the Nrf2 pathway is functioning properly.

The results in Figure 8 indicate that TAC levels in diabetic mice's kidneys were remarkably lower than in healthy mice ($P<0.001$). Furthermore, examining MDA levels showed that diabetic mice had higher levels than control mice ($P<0.001$). The decrease in TAC could be related to the high level of reactive oxygen species that can inhibit the function of anti-oxidant enzymes. In the harmaline group, TAC increased relative to the diabetic group ($P<0.001$), and in the metformin group, $P<0.05$. Harmaline had a greater effect on increasing anti-oxidant capacity ($P<0.05$). In diabetic mice treated with Harmaline and metformin, there was a decrease in MDA levels ($P<0.001$), and the reduction in TAC was counteracted. The improved anti-oxidant status in the kidney tissues of these mice could be due to the higher levels and increased activity of the Nrf2 protein in these groups.

Discussion

The present study demonstrated the protective efficacy of Harmaline against diabetes-induced kidney injury. The findings suggest that Harmaline's anti-oxidant and anti-

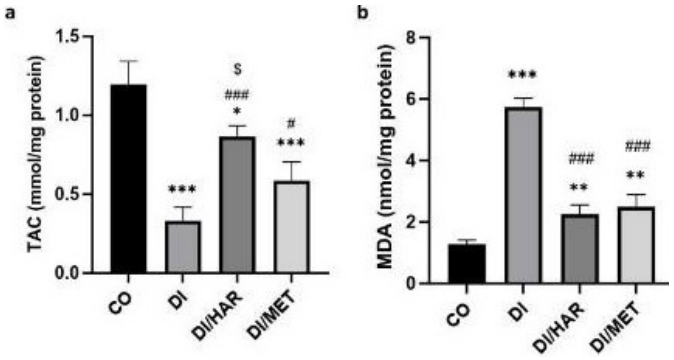

Figure 8. Effects of HAR on:
a) Mouse TAC; and b) MDA of the mouse kidney tissue
CO group, DI control group, DI treated with HAR, and DI treated with MET (positive control group).
Data are presented as mean±SEM (n=6/group). Statistical analysis was performed using one-way ANOVA followed by Tukey's *post hoc* test. * Compared to CO and # compared to DI. $ indicates comparison between DI/HAR and DI/MET (positive control) groups. 1, 2, and 3 symbols represent statistically significant differences with $P<0.05$, $P<0.01$, and $P<0.001$, respectively
HAR: Harmaline; CO: Control; DI: Diabetic; MET: Metformin; TAC: Total anti-oxidant capacity; MDA: Malondialdehyde

inflammatory properties may help attenuate biochemical, molecular, and histopathological alterations associated with diabetic renal dysfunction. Consistent with previous studies in experimental diabetes models (22, 23), NA/STZ injections were used to induce type 2 diabetes. Elevated FBG, glucosuria, and polyuria in the diabetic group confirmed successful induction of type 2 diabetes. As reported by Noei Razliqi *et al.*, hyperosmotic diuresis resulting from excessive glucose excretion can lead to loss of water and essential electrolytes (24). In the present study, diabetic mice exhibited reduced plasma sodium and increased urinary sodium compared with controls, consistent with findings by Huang *et al.* (25). Although insulin promotes renal sodium reabsorption (26), diabetes-associated osmotic diuresis, tubular dysfunction, and inflammatory alterations may collectively contribute to impaired sodium handling. Harmaline attenuated these electrolyte disturbances, suggesting improved renal tubular regulation.

Increased urinary sodium and potassium excretion has been associated with renal dysfunction under diabetic conditions (27), indicating altered tubular electrolyte handling in diabetes. The observed imbalance in potassium homeostasis, characterized by elevated serum potassium and increased urinary potassium excretion, may result from an adaptive response to hyperglycemia-induced osmotic diuresis and enhanced distal tubular potassium secretion. Elevated serum potassium may also be associated with impaired insulin-mediated cellular potassium uptake and altered renal potassium regulation. In addition to these metabolic mechanisms, diabetes-induced renal inflammation, particularly NF-κB signaling, which leads to increased expression of pro-inflammatory mediators such as TNF-α, IL-1β, and IL-6, and alterations in TGF-β expression, may contribute to impaired electrolyte homeostasis by affecting renal transport processes. Clinically, increased serum potassium levels are important because hyperkalemia can impair cardiac conduction and increase the risk of potentially life-threatening arrhythmias (28). However, since cardiac electrophysiological parameters were not assessed in the present study, further investigations are required to determine whether these electrolyte disturbances are associated with functional

cardiac abnormalities. Although impaired insulin action and reduced Na^+/K^+-ATPase activity may contribute to disturbed electrolyte distribution (24), the association between electrolyte abnormalities and inflammatory activation highlights the potential role of renal inflammation in diabetes-induced tubular dysfunction. The improvement of sodium and potassium disturbances following Harmaline treatment may therefore be partly attributed to its ability to modulate renal inflammatory responses and preserve tubular function.

Diabetic mice exhibited decreased plasma albumin, increased urinary albumin, elevated plasma creatinine, reduced urinary creatinine, increased BUN, and increased urinary urea, consistent with renal functional impairment. Albuminuria is an important indicator of kidney injury, and elevated blood creatinine and BUN levels reflect impaired renal function (29). Harmaline treatment significantly attenuated these alterations, suggesting protective effects against diabetes-associated renal injury.

To further evaluate renal function, DN indices, including fractional excretion of sodium and potassium, 24-hr albumin excretion, urinary albumin-to-creatinine ratio, and TGF-β levels, were assessed. Harmaline reversed the elevations in FENa+, FEK+, urinary albumin, UACR, and TGF-β in diabetic mice, suggesting improved renal function and reduced fibrotic signaling. Increased FENa+ and FEK+ have been reported in type 2 diabetes (27). This is attributed to impaired insulin production or function, which reduces the effectiveness of the sodium-potassium pump and leads to further loss of these electrolytes (30). Elevated albuminuria and UACR are established markers of diabetic nephropathy (31), while TGF-β plays a central role in renal fibrosis (32). Histopathological findings, including mesangial expansion, glomerulosclerosis, and tubular injury in diabetic mice, were consistent with previous descriptions of diabetic renal damage (33). Oxidative stress and inflammation are recognized contributors to these structural alterations (34, 35).

Our findings showed increased activation of the RAS in diabetic mice, evidenced by elevated ACE1 and AT1R mRNA expression and angiotensin II levels. Harmaline significantly reduced these parameters. Dysregulation of the intrarenal RAS is a key mechanism in DN (36). Hyperglycemia enhances Ang I and Ang II production, leading to increased ROS generation, NF-κB activation, and up-regulation of pro-inflammatory cytokines such as TNF-α and IL-6 (37, 38). Ang II also promotes TGF-β–mediated fibrosis (39) and contributes to insulin resistance and albuminuria in diabetes (40). The binding of Ang II to AT1R induces vasoconstriction, oxidative stress, hypertrophy, and fibrosis (13). Elevated ACE1 and Ang II levels have been documented in diabetic kidneys (41, 42). Therefore, suppression of the ACE1/AngII/AT1R axis by Harmaline may represent one mechanism underlying its renoprotective effects.

High concentrations of inflammatory markers in the blood or urine were considered among the most important signs of diabetic kidney disease, demonstrating a strong link between inflammation and kidney problems in people with diabetes (33). The study observed that NF-κB activation, mediated by increased miR-125b, can exacerbate inflammation, whereas Harmaline was associated with reduced expression. A study confirming our results has shown that one of the most important roles of microRNAs in the body is controlling gene function. For example, miR-125b influences the NF-kB gene by binding to it (43). Interestingly, miR-125b's role in DN extends beyond simply regulating NF-kB. Huang *et al.* demonstrated that miR-125b is up-regulated in renal tubular epithelial cells under high-glucose conditions and targets ACE2, leading to its down-regulation; given that ACE2 normally degrades Ang II and counterbalances the classical RAS axis, this suppression by miR-125b may shift the RAS balance toward enhanced ACE/Ang II/AT1R activity and contribute to increased RAS activation in DN (44). Since high NF-κB activity leads to increased proteinuria, reducing it could be a promising approach to treating diabetic kidney disease (45). The use of NF-κB inhibitors has been suggested as a potential therapeutic strategy for DN (46). Increased NF-kB activity in the present study led to increased levels of the inflammatory proteins TNF-α and IL-1β in diabetic mice, and Harmaline ameliorated this increase. In other experimental studies, the production of proinflammatory cytokines, such as TNF-α and IL-1β, by kidney cells activates various signaling pathways and alters cellular function. These changes ultimately lead to cell damage or death (47, 48). High levels of TNF-α exacerbate kidney damage caused by these two factors by producing local oxygen-free radicals and inflammatory factors (49). Research indicates that higher amounts of IL-1β are linked to diabetes and more inflammation in mice that have diabetes (50). In these mice, TNF-α and IL-1β harm the kidney's podocytes by lowering the levels of glomerular VEGF and eNOS and by changing hemodynamic factors. These changes cause and worsen inflammatory damage in DN (51). In line with our study, Reddy *et al.* have linked the pro-inflammatory cytokine IL-6 to the development of DN and have noted higher levels of this cytokine in individuals suffering from the condition. IL-6 is involved in both inflammation and the regulation of blood glucose levels. This makes it a promising biomarker and therapeutic target for DN and type 2 diabetes (52). These experimental studies are aligned with our observations, and Harmaline was able to improve all inflammatory factors evaluated in diabetic mice. This is a significant finding that focusing on the miR125b/NF-kB pathway is effective in treating type 2 diabetes.

Oxidative stress is a critical contributor to the pathogenesis of diabetic kidney injury, resulting from excessive reactive oxygen species (ROS) generation and an inadequate anti-oxidant response (53). Persistent oxidative imbalance can impair cellular function and promote renal damage by affecting key kidney cells, thereby accelerating the progression of DN (54). In the present study, the miR-200a/Keap1/Nrf2/Nqo1 pathway was modulated in diabetic mice and the harmaline group. Activation of Nrf2 signaling enhances the expression of anti-oxidant defense genes and promotes restoration of redox homeostasis, thereby limiting oxidative stress-mediated renal injury (55). Keap1 acts as a negative regulator of Nrf2 by sensing oxidative stress and facilitating Nrf2 degradation under basal conditions, preventing the transcriptional activation of anti-oxidant response genes (56, 57). In the present study, Harmaline decreased Keap1 expression and increased miR-200a levels, which may contribute to enhanced Nrf2 activation and improved anti-oxidant capacity in diabetic kidneys. These findings are consistent with previous evidence demonstrating the regulatory relationship between miR-200a and oxidative stress through modulation of the Keap1/Nrf2 pathway (58). Previous studies have also reported that natural anti-

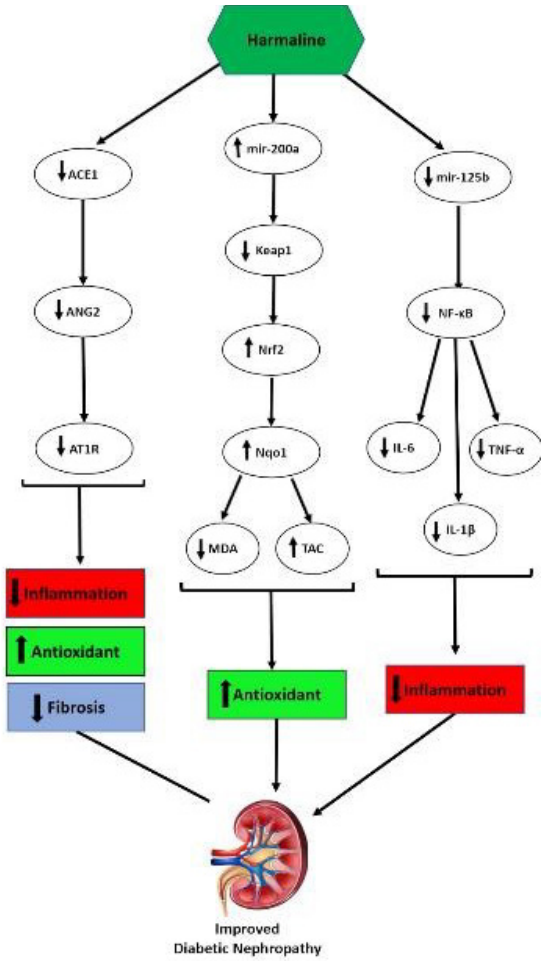

Figure 9. Proposed mechanistic pathways underlying the renoprotective effects of Harmaline in diabetic nephropathy

oxidants can increase miR-200a expression, suppress Keap1 and subsequently enhance Nrf2-mediated anti-oxidant responses, providing protection against oxidative damage (59, 60). Furthermore, Nqo1, a downstream anti-oxidant enzyme regulated by the Nrf2-Keap1 pathway, was elevated in diabetic mice, consistent with previous observations of increased Nqo1 expression in diabetic mice (61). Harmaline was also effective at further increasing Nqo1 expression. Previous studies have also reported that natural anti-oxidants can attenuate diabetes-induced increases in MDA levels and reductions in TAC, indicating an improvement in oxidative balance. Similar alterations, including increased renal MDA and decreased TAC, have been reported in diabetic animal models (62). Moreover, El Tabaa *et al.* demonstrated that TAC enhancement mitigates STZ-induced DN in mice (63). Collectively, these findings suggest that Harmaline may exert renoprotective effects by modulating the miR-200a/Keap1/Nrf2/Nqo1 axis and enhancing endogenous anti-oxidant defense mechanisms.

In recent years, reducing oxidative stress has become a key approach in treating DN. The anti-oxidant properties of medicinal plants have attracted interest for their potential to manage diabetes and its complications, including DN (64). Our findings indicate that Harmaline improves DN. The protective effects of Harmaline are partly due to its ability to repair tissue damage, exhibit anti-oxidant activity, and reduce inflammation. These findings align with other studies that have reported the anti-oxidant and anti-inflammatory effects of Harmaline (18, 65). Given its ability to lower oxidative stress and inflammation, it is reasonable to suggest that this compound, derived from the plant P. harmala, may play a beneficial role in the management of nephropathy. This is the first study demonstrating that Harmaline dual-modulates the miR-125b/NF-κB inflammatory axis and the miR-200a/Keap1/Nrf2 anti-oxidant pathway in experimental DN. The present findings suggest a potential mechanistic model underlying the renal protective effects of Harmaline in DN. As shown in Figure 9, Harmaline exerts its effects by simultaneously inhibiting the ACE1/AngII/AT1R axis and miR-125b/NF-κB/TNF-α, IL-6, and IL-1β, and by activating the miR-200a/Keap1/Nrf2 anti-oxidant pathway. These effects collectively reduce oxidative stress, inflammation, and fibrosis, ultimately reducing diabetic kidney damage.

Conclusion

Harmaline improved glycemic status, attenuated markers of renal dysfunction, reduced oxidative stress and inflammatory mediators, and modulated key molecular pathways in the NA/STZ diabetic model. These findings suggest that Harmaline warrants further investigation as a potential therapeutic candidate to mitigate diabetic kidney injury. However, mechanistic validation and clinical investigations are required to confirm its translational applicability.

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Ethical Approval and Consent to Participate

The animals were treated in accordance with the guidelines of Animal Care and approved by the Animal Ethics Committee of Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran (IR.AJUMS.AEC.1403.012).

Authors' Contributions

F M performed experiments, collected data, and prepared the draft manuscript. A A designed the experiments, provided supervision, secured funding, analyzed and interpreted the results, and critically revised or edited the article. KH H provided methodological support and laboratory resources and was involved in the final review of the manuscript. S A M was responsible for verifying the associated analyses. M R critically revised or edited the article. F N contributed by taking photographs of the tissue and examining and evaluating the histological changes. F M, A A, KH H, S A M, M R, and F N approved the final version to be published.

Conflicts of Interest

The authors declare no competing interests or financial relationships that could influence the work reported in this article.

Declaration

We have not used any AI tools or technologies to prepare the manuscript. An AI-assisted language-editing tool (Grammarly) was used solely for grammar correction and to improve clarity. The authors are fully responsible for the scientific content, data analysis, interpretation, and conclusions presented in this manuscript.

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