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COMPARATIVE EFFECTS OF WILD HYPERICI HERBA AND *HYPERICUM PERFORATUM* L. HAIRY ROOT EXTRACTS ON RENAL CARBOHYDRATE METABOLISM AND OXIDATIVE STRESS IN STZ-INDUCED DIABETIC RATS

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ABSTRACT

Chronic hyperglycemia-induced imbalances in renal carbohydrate metabolism and oxidative stress contribute to diabetic kidney complications, highlighting the urgent need for therapies targeting both pathways. This study evaluates nephroprotective effects of *Hypericum perforatum* L. extracts-wild Hyperici herba (HH) and hairy root (HR)-in streptozotocin-induced diabetic Wistar rats. Male Wistar rats received single intraperitoneal streptozotocin injection (45 mg/kg body weight) with diabetes confirmed by fasting blood glucose >15 mmol/L. Diabetic rats were treated with daily oral doses of HH or HR extracts for 14 consecutive days. Renal carbohydrate metabolism was assessed through glycogen content, glucose levels, glucose-6-phosphate content, glucose-6-phosphatase and glucose-6-phosphate dehydrogenase activities. Oxidative stress markers evaluated included catalase, superoxide dismutase, glutathione reductase, glutathione peroxidase activities, total glutathione levels, and malondialdehyde (MDA) content. Diabetes induced 1.56-fold renal glucose increase, 1.7-fold glycogen accumulation, 47.5% higher glucose-6-phosphatase activity, 39.4% lower glucose-6-phosphate dehydrogenase activity, 44% catalase reduction, and elevated MDA levels. Both extracts significantly improved these parameters; HH reduced glucose-6-phosphatase by 33.1%, restored dehydrogenase by 61.5%; HR showed superior effects (37.5% and 67.3% respectively) plus normalized glycogen and enhanced catalase above controls. Glibenclamide had limited effects. Xanthone-enriched HR extract demonstrated superior nephroprotection versus HH and glibenclamide through comprehensive restoration of carbohydrate metabolism and antioxidant defenses in diabetic nephropathy.

Key words: xanthones, experimental diabetes, antioxidant defense, carbohydrate metabolism, kidney

INTRODUCTION

Chronic hyperglycemia is a hallmark of diabetes and plays a central role in the diabetic nephropathy (DN) development, a major microvascular complication affecting approximately 45% of individuals with Type 1 and Type 2 diabetes (1). DN is the leading cause of end-stage renal disease worldwide (2), driven by metabolic disturbances and oxidative stress. In the diabetic kidney, disruptions in carbohydrate metabolism are evident, with renal gluconeogenesis contributing up to 40% of endogenous glucose production and excessive glucose uptake leading to glycogen accumulation (3, 4). This altered metabolic state is further exacerbated by insulin deficiency, which enhances gluconeogenesis and impairs glucose utilization, ultimately promoting glycogen overload.

At the molecular level, hyperglycemia triggers the formation of advanced glycation end products (AGEs) and increases reactive oxygen species (ROS)

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production, leading to oxidative stress and cellular dysfunction (5). The kidney's high mitochondrial density, a primary site of ROS generation (6), and its abundance of oxidation-prone polyunsaturated fatty acids (7) make it particularly susceptible to oxidative damage. This oxidative imbalance plays a key role in diabetic complications that target the kidneys by impairing antioxidant defense mechanisms, depleting glutathione levels, and disrupting redox homeostasis (8). Given the intricate relationship between dysregulated carbohydrate metabolism and oxidative stress in the kidney in diabetic state, there is an urgent need for therapeutic strategies that simultaneously target both metabolic and oxidative pathways to provide nephroprotection.

Hypericum perforatum L. has been widely studied for its antioxidant properties, attributed to its rich phenolic composition (9, 10, 11). Flavonoids within the plant are potent free radical scavengers (12) that accumulate in renal cells and exert dose-dependent protective effects against oxidative stress (13). *In vivo* studies have confirmed the antioxidative potential of *H. perforatum* extracts, demonstrating their ability to chelate metal ions and inhibit radical-producing enzymes (11, 13, 14, 15). Although research on the direct effects of *H. perforatum* extracts on renal carbohydrate metabolism is limited, bioactive phenolic compounds have been shown to regulate key metabolic enzymes involved in glucose homeostasis (16).

However, the phenolic profile of wild-growing *H. perforatum* (Hyperici herba, HH) is highly variable due to environmental factors such as climate, soil composition, and pathogenic exposure, which pose challenges for therapeutic consistency (17, 18). To address these limitations, *Agrobacterium rhizogenes*-mediated transformation has been employed to generate hairy root (HR) cultures of *H. perforatum*, providing a stable and enhanced source of bioactive metabolites (19). Notably, HR extracts are enriched in xanthones—such as mangiferin and its derivatives—which have demonstrated both carbohydrate-modulating and antioxidative effects *in vitro* and *in vivo* (20, 21, 22, 23, 24).

Despite the promising potential of *H. perforatum* HR extract, its specific effects on oxidative stress and carbohydrate metabolism in the diabetic kidney remain underexplored. Therefore, this study aims to evaluate and compare the antioxidative and carbohydrate metabolism-modulating effects of HH and HR extracts in the kidneys of streptozotocin-induced diabetic rats. By assessing their impact on gluconeogenesis, glycogen metabolism, and oxidative stress markers, this study seeks to determine whether HR extract offers superior nephroprotective benefits relative to its wild-type counterpart.

MATERIAL AND METHODS

Plant material and extract preparation

Wild specimens of *H. perforatum* (voucher no. 060231) were collected in the Republic of North Macedonia. The aerial parts (flower shoots, designated as Hyperici herba, HH) were air-dried in the absence of light, and then milled into a fine powder using a laboratory grinder. Phenolic compounds were extracted with 80% aqueous methanol (v/v). The extracts were centrifuged at 15,000 g for 15 min, and the supernatants were evaporated to dryness under reduced pressure (50 mbar) at room temperature using a rotary evaporator. The dried HH extract was reconstituted in 0.3% carboxymethylcellulose (CMC, w/v in distilled water) at a final concentration of 20 mg/mL prior to oral administration. The extraction yield of the HH extract was 45% (w/w, dry extract relative to dry plant material). In addition, *H. perforatum* seeds were used to produce *in vitro* seedlings that were transformed via *Agrobacterium rhizogenes* A4 to establish hairy root (HR) cultures. After one month of growth in liquid MS/B5 medium, the HR cultures were harvested and lyophilized to complete dryness. The extraction of phenolic constituents was performed with 80% methanol (v/v). Extraction of phenolic compounds from lyophilized HR biomass (0.2 g) was performed with 80% (v/v) methanol using an ultrasonic bath for 15 min, followed by centrifugation at 15,000 g for 15 min. The resulting supernatants were evaporated to dryness under reduced pressure (50 mbar) at room temperature using a rotary evaporator.

The dried HR extract was dissolved in 0.3% CMC (w/v in distilled water) at a concentration of 20 mg/mL prior to administration to animals. The extraction yield of the HR extract was 65% (w/w, dry extract relative to dry biomass).

Animals

Adult Wistar rats (200–250 g, 14–16 weeks old) were sourced from the Vivarium of the Faculty of Natural Sciences and Mathematics, Skopje. Rats were housed under standard conditions (25±2 °C; 55±10% relative humidity; 12 h light/dark cycle) with free access to water and a standard pellet diet

(20% protein, 30% carbohydrate, 9% lipid, 2.5% cellulose, 10% water; 310 kcal). All procedures involving animals were approved by the Animal Ethics Committee of the University "Ss. Cyril and Methodius", Skopje, North Macedonia (Approval No. 03-2323/2) and conducted in accordance with ethical guidelines such as CIOMS and ICLAS. The study also complied with Directive 2010/63/EU, and ILAR standards.

Experimental design

The STZ-induced diabetes model was employed as previously described (25). Diabetes was induced experimentally by a single intraperitoneal injection of freshly prepared streptozotocin (STZ; Sigma Aldrich, Bangalore) at 45 mg/kg b.w. in cold 0.1 M citrate buffer (pH 4.5). Diabetic status was confirmed three days later, and animals with fasting blood glucose levels exceeding 15 mmol/L (measured seven days post-injection) were classified as diabetic and included in the study. Glibenclamide (Glb; Alkaloid AD Skopje) administered at a dose of 2.5 mg/kg b.w. served as a positive control, because its glucose-lowering effect is well established (26), allowing comparison with the experimental HH and HR extracts. For evaluation of the effects of the HH and HR extract treatments in the kidneys, both healthy and diabetic rats (n = 8 animals per group) were randomly allocated to the following groups: C: Normal control, treated with water; CH: Healthy rats receiving HH extract (200 mg/kg in 0.3% CMC); CHR: Healthy rats receiving HR extract (200 mg/kg in 0.3% CMC); D: Diabetic control, treated with 0.3% CMC; DGlb: Diabetic rats receiving Glb (2.5 mg/kg); DH: Diabetic rats receiving HH extract (200 mg/kg in 0.3% CMC); DHR: Diabetic rats receiving HR extract (200 mg/kg in 0.3% CMC).

Treatments were administered once daily via oral gavage for 14 consecutive days after an 8-hour fast. At the end of the treatment period, animals were anesthetized with sodium thiopental (45 mg/kg), administered in accordance with EC Directives 86/609/EEC and 2010/63/EU, and euthanized by standard laparotomy procedure. Kidneys were quickly excized, rinsed in cold saline and snap-frozen in liquid nitrogen until further analysis.

Analytical methods

Kidney homogenates were prepared in appropriate buffers for enzyme activity assays. Glucose-6-phosphatase (G6Pase) activity was measured using the method described by Hers (27), while glucose-6-phosphate dehydrogenase (G6PDH) activity was determined using a modified protocol from Sigma-Aldrich. In these assays, spectrophotometric measurements were used to quantify the release of inorganic phosphate as per Fiske and Subbarow (28). Enzyme activities were expressed in nmol P_i/min/mg protein, with protein concentrations determined by the Lowry method (29) using bovine serum albumin as the standard. Glycogen, glucose, and glucose-6-phosphate levels were quantified as per Kepler and Decker (30).

Total glutathione levels were quantified with a commercial Glutathione Assay Kit (Sigma-Aldrich, Collegeville, PA, USA). Activities of glutathione peroxidase (GPx) and glutathione reductase (GR) were assessed using modified versions of commercial kits from Sigma-Aldrich with protocols adjusted for 10% kidney homogenates and microplate detection by modifying sample dilution and incubation time for reaction linearity. Catalase (Cat) activity was determined using Aebi's method (31), superoxide dismutase (SOD) activity was measured according to the method of Marklund and Marklund (32), and malondialdehyde levels were measured following Zeb and Ullah (33).

Statistical analysis

Data are expressed as mean±SD. Statistical comparisons among groups were performed using one-way ANOVA followed by Tukey's post hoc test (GraphPad Prism, version 9), with $p<0.05$ indicative of statistical significance.

RESULTS

Carbohydrate metabolism

Fig. 1 shows the changes in carbohydrate metabolism in the kidney of healthy and diabetic rats treated with HH and HR. Treatment with HH and HR extracts in healthy rats did not lead to significant changes in glucose levels (A), glucose-6-phosphate levels (B), or glycogen content (E). Similarly, there were no significant alterations observed in the activity of glucose-6-phosphatase (C) or glucose-6-phosphate dehydrogenase (D).

However, in diabetic rats, there was a notable increase in glucose levels (A) by 1.56-fold and glycogen content (E) by 1.7-fold compared to healthy controls. Furthermore, diabetic rats exhibited significant increases in glucose-6-phosphatase activity (C) by 47.5% and glucose-6-phosphate content (B) by 80.9%, alongside a significant decrease in glucose-6-phosphate dehydrogenase activity (D) by 39.4%.

Conversely, treatment with HH and HR extracts in diabetic rats resulted in substantial reductions in glucose levels (A) by 48% and 54.9%, respectively, compared to diabetic controls. Additionally, there were significant decreases in glycogen content (E), with the HR extract showing a more pronounced effect (reduction by 52.4%, compared to 23.7% with HH). Treatment with HH and HR extracts in diabetic rats also led to decreases in glucose-6-phosphatase activity (C) by 33.1% and 37.5%, respectively, and increases in glucose-6-phosphate dehydrogenase activity (D) by 61.5% and 67.3%, respectively ($p<0.05$ for both).

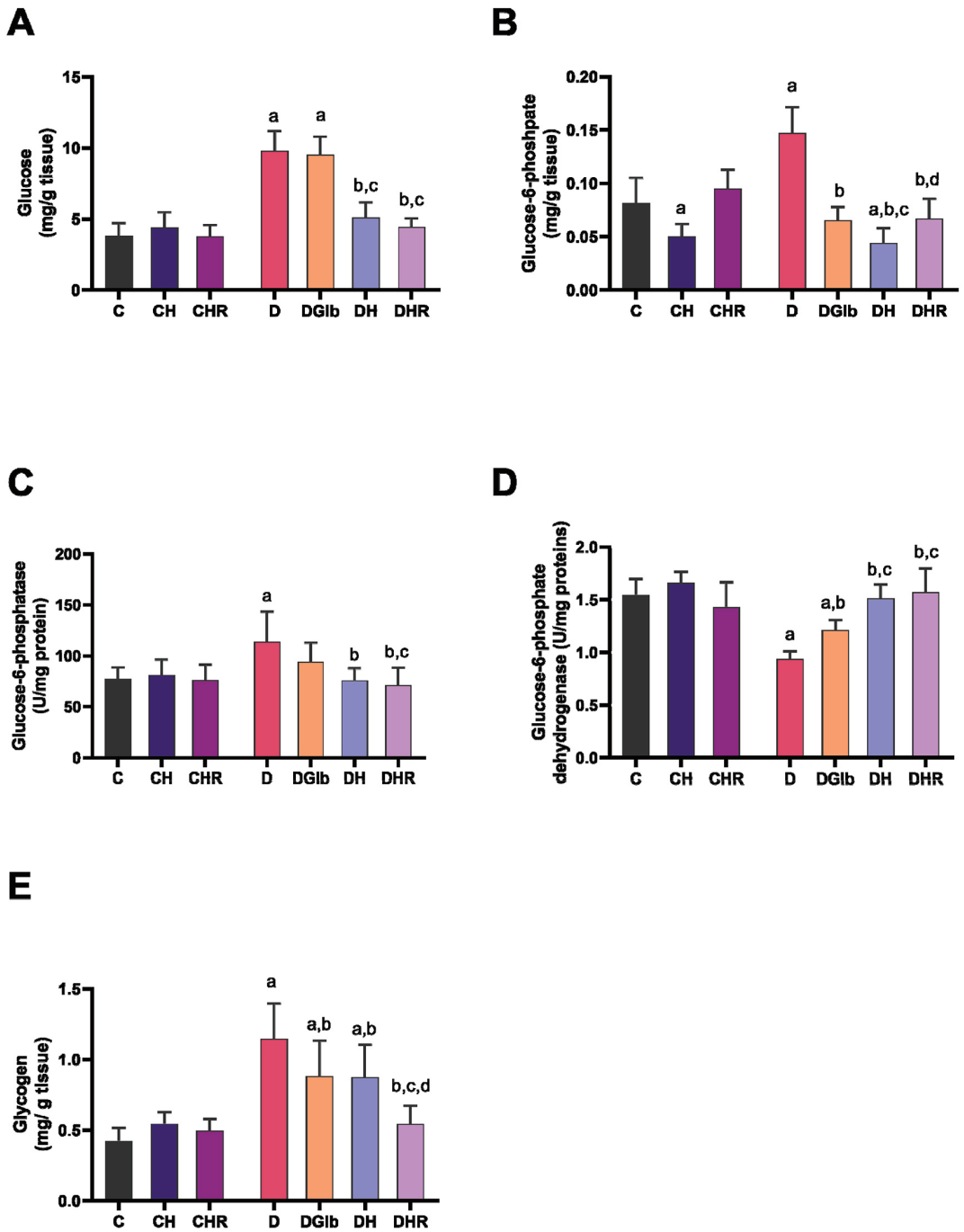

Figure 1. Changes in glucose levels (A), glucose-6-phosphate levels (B), glucose-6-phosphatese activity (C), glucose-6-phosphate dehydrogenase activity (D), glycogen (E) in healthy and diabetic rats treated with HH and HR extracts. Data are presented as the mean±SD (n=8 rats per group). Significant differences ($p<0.05$): a-compared to C; b-compared to D; c-compared to DGlb; d-compared to DH

Additionally, both treatments resulted in a decrease in glucose-6-phosphate levels (B) in the kidneys, with the HH extract demonstrating a more pronounced effect than the HR extract ($p<0.05$).

Furthermore, treatment with glibenclamide did not significantly affect glucose levels, but led to a significant decrease in glycogen content by 23.2% ($p<0.05$). Additionally, the treatment with glibenclamide resulted in a significant decrease in glucose-6-phosphate content (B) by 55.4% and a significant increase in glucose-6-phosphate dehydrogenase activity (D) by 29.1% ($p<0.05$).

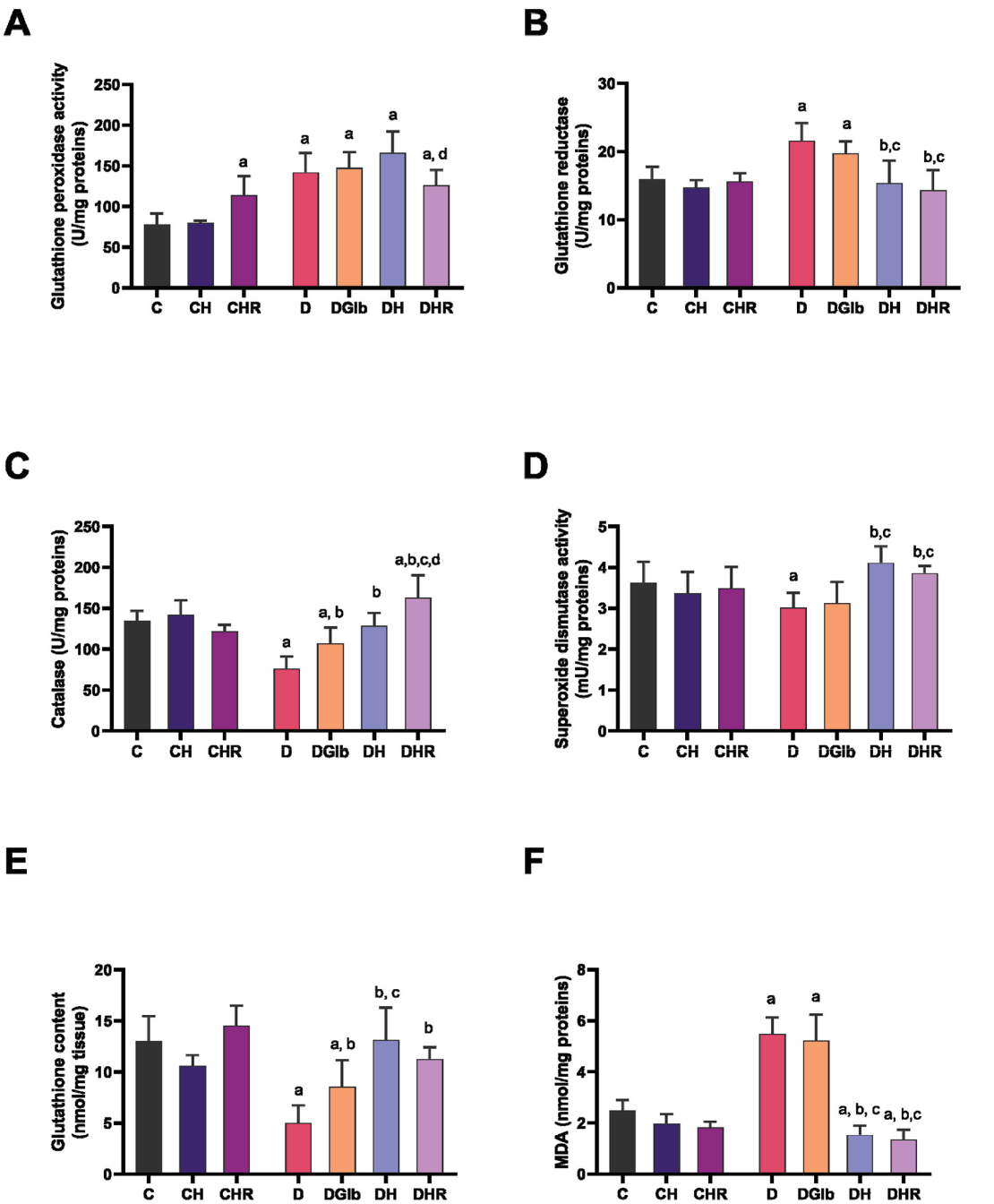

Figure 2. Changes in glutathione peroxidase activity (A), glutathione reductase activity (B), catalase activity (C), superoxide dismutase activity (D), glutathione content (E) and MDA levels (F) in healthy and diabetic rats treated with HH and HR extracts. Data are presented as the mean±SD (n=8 rats per group). Significant differences ($p<0.05$): a-compared to C; b-compared to D; c-compared to DGlb; d-compared to DH

Oxidative stress parameters

Fig. 2 shows the changes in oxidative stress parameters in the kidney of healthy and diabetic rats treated with HH and HR. In the case of healthy rats, the treatment with HH and HR extracts did not significantly alter catalase (C), superoxide dismutase (D), or glutathione reductase activities (B), nor GSH (E) and MDA (F) levels in the kidney. Interestingly, HR-but not HH treatment, led to a significant increase in glutathione peroxidase activity (A) in the kidneys of healthy rats.

Experimental diabetes led to a significant decrease in the activities of catalase (C) and superoxide dismutase (D), by 44% and 18%, respectively. Additionally, there was a substantial increase in glutathione reductase activity (B) by 35.6% and glutathione peroxidase activity (A) by 69.1%, accompanied by reduced GSH content (E), and elevated MDA levels (F) in the kidneys compared to the control group.

Both HH and HR treatments significantly increased the activities of catalase (C) and superoxide dismutase (D), with HR treatment elevating catalase activity above control levels ($p<0.05$). Additionally, HH and HR extracts markedly reduced glutathione reductase activity by 28.8% and 33.5%, respectively (B) while simultaneously increasing GSH content (E) in the kidneys of diabetic rats. However, neither treatment produced significant changes in glutathione peroxidase activity (A), but insignificant trends towards reduction were achieved with HR treatment, and there was a significant 24.1% difference in glutathione peroxidase activity between HH and HR treatment ($p<0.05\%$). Notably, both extracts led to a substantial reduction in MDA levels (F), bringing them below the control values.

Treatment with glibenclamide did not significantly affect the activity of glutathione peroxidase (A), glutathione reductase (B), and superoxide dismutase (E), or MDA content (F) compared to the diabetic group. However, glibenclamide treatment significantly increased catalase activity (C) and glutathione content (E) in diabetic rats, though not to the levels observed in the control group.

DISCUSSION

This study aimed to evaluate and compare the effects of a two-week treatment with Hyperici herba (HH) and *Hypericum perforatum* L. hairy root (HR) extracts on carbohydrate metabolism and oxidative stress in the kidney of diabetic rats. These effects are largely attributed to the presence of bioactive metabolites, which can modulate key metabolic and oxidative stress-related pathways. Chromatographic analysis revealed that HH extract is rich in various phenolic compounds, including chlorogenic acid, flavan-3-ols, hyperoside, rutin, biapigenin, and hyperforin (34), whereas HR extract is characterized by five groups of phenolic compounds, such as phenolic acids, flavan-3-ols, flavonol glycosides, dihydrochalcones, and xanthones (35). Among the identified compounds, mangiferin, γ-mangostin isomers, and trihydroxy-1-methoxy-C-prenylxanthone were found as dominant xanthones in *H. perforatum* HR extracts (35).

Diabetes induced significant disturbances in renal carbohydrate metabolism, as evidenced by increased glucose levels, glycogen accumulation, and enhanced glucose-6-phosphatase activity. The 1.56-fold increase in glucose levels and 1.7-fold rise in glycogen content suggest impaired glucose utilization and excessive glycogen deposition in diabetic kidneys. This is likely driven by insulin deficiency—previously documented in our work (26), which promotes gluconeogenesis while the kidney's insulin-independent glucose uptake contributes to glycogen overload. Additionally, the observed 39.4% reduction in glucose-6-phosphate dehydrogenase activity points to a diminished pentose phosphate pathway, which is known to weaken antioxidant defenses and exacerbate oxidative stress (36, 37). Consistently, our findings indicate oxidative stress in diabetic kidneys, as reflected by the significant reductions in catalase (44%) and superoxide dismutase (18%) activity, coupled with elevated lipid peroxidation, evidenced by increased MDA levels. The altered glutathione redox balance further confirms oxidative imbalance, with increased glutathione reductase and glutathione peroxidase activities failing to compensate for the substantial depletion of GSH. The persistent decline in GSH, a crucial marker of redox homeostasis (38), suggests an inadequate antioxidant response, leaving the diabetic kidney under sustained oxidative stress, ultimately leading to progressive cellular damage and dysfunction. Previously reported effects of HH and HR extracts reduced plasma glucose concentrations in STZ-diabetic rats by 70.1% and 72.7%, respectively, after 14-day treatment, achieving normoglycemia with no significant difference from controls (26). Treatment with HH extract significantly attenuated renal gluconeogenesis in diabetic rats, as evidenced by a 33.1% reduction in glucose-6-phosphatase activity

compared to diabetic controls. This effect is likely mediated by its insulinotropic properties (39) and the inhibitory actions of bioactive compounds such as chlorogenic acid and rutin on key gluconeogenic enzymes (40, 41). Additionally, HH extract improved glucose-6-phosphate dehydrogenase activity by 61.5%, contributing to the restoration of the pentose phosphate pathway, which is crucial for maintaining redox homeostasis (16). However, despite reducing glycogen accumulation by 23.7%, glycogen levels remained elevated relative to healthy controls, potentially due to the inhibitory effects of flavonoids, particularly quercetin, on glycogen phosphorylase (42). Compared to HH, HR extract demonstrated a more pronounced effect on carbohydrate metabolism, with a 37.5% reduction in glucose-6-phosphatase activity and a 67.3% increase in G6PD activity. Notably, HR treatment led to a greater reduction in glycogen content (52.4% vs. 23.7% for HH), suggesting superior regulation of glycogen metabolism. These differences may be attributed to the presence of xanthones such as mangiferin and quercetin, which have been reported to inhibit gluconeogenic enzymes more effectively (21, 22). Both HH and HR extracts exhibited strong antioxidant effects, significantly enhancing catalase and superoxide dismutase activities while reducing glutathione reductase activity and increasing GSH levels. However, HR extract elicited a more pronounced increase in catalase activity, restoring it to levels above those of healthy controls, suggesting a more effective reduction of hydrogen peroxide-mediated oxidative stress. The stronger antioxidant effects of HR may be attributed to its unique composition, particularly the presence of mangiferin, (-)-epicatechin, and proanthocyanidins. Mangiferin has been shown to enhance catalase activity and inhibit ROS production (43), while (-)-epicatechin and proanthocyanidin dimers have been reported to increase catalase activity *in vivo* (24). Additionally, mangostin enhances superoxide dismutase activity, further supporting the antioxidant potential of HR extract (23). Both extracts effectively restored the GSH pool and improved the glutathione redox cycle by reducing glutathione reductase activity, indicating enhanced recycling of oxidized glutathione and improved redox balance. However, neither treatment significantly altered glutathione peroxidase activity, although HR treatment exhibited a nonsignificant trend towards reduction and resulted in a significant 24.1% difference in glutathione peroxidase activity between the HH- and HR-treated groups. This suggests that HR extract may offer a slight advantage in modulating GPx activity, potentially due to its more potent antioxidant composition. The significant increase in glutathione peroxidase activity observed in the CHR group compared to controls suggests that HR extract supplementation enhances renal antioxidant capacity even under non-diabetic conditions, likely reflecting an adaptive upregulation of endogenous antioxidant defenses rather than a response to oxidative stress (23). Moreover, both HH and HR extracts markedly reduced lipid peroxidation, as reflected by decreased MDA levels, with reductions surpassing those seen in control rats. This further underscores their role in mitigating oxidative damage in diabetic kidneys. In contrast, glibenclamide (2.5 mg/kg b.w.) showed no significant effects on renal antioxidant enzyme activities or tissue glucose parameters, consistent with its mechanism as a pancreatic insulin secretagogue that lowers blood glucose via ATP-sensitive K+ channel inhibition without directly targeting peripheral oxidative stress pathways (44, 45, 46). Unlike the extracts, which achieved normoglycemia (70.1-72.7% reductions) while comprehensively restoring renal metabolism and redox balance, glibenclamide's glycemic improvement (38.5% reduction) lacked tissue-protective benefits, highlighting the superior therapeutic potential of HH and HR extracts. Overall, while both HH and HR extracts significantly improved renal metabolic and oxidative stress parameters in diabetic rats, HR extract demonstrated superior efficacy in regulating glycogen metabolism, enhancing antioxidant enzyme activity, and modulating oxidative stress markers. These findings suggest that HR extract may provide a more comprehensive protective effect against diabetes-induced renal dysfunction.

CONCLUSION

Both HH and HR extracts effectively modulated renal carbohydrate metabolism and improved antioxidant defenses in diabetic rats. However, HR extract was more efficient in normalizing glycogen accumulation and enhancing catalase activity, thereby offering superior protection against hydrogen peroxide-mediated oxidative damage. These differences likely arise from the distinct phenolic compositions of the extracts, with HR's xanthone-rich profile exerting a dominant influence on both metabolic and antioxidative pathways. Collectively, these findings highlight HR extract as a promising therapeutic agent for

mitigating renal metabolic disturbances in diabetes, warranting further investigation into its underlying mechanisms and clinical potential.

CONFLICT OF INTEREST

The authors declare that they have no financial or non-financial conflict of interest regarding authorship and publication of this article.

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AUTHORS' CONTRIBUTION

ER, EM, CT, BM and SDK conceived and designed the study, supervised the experiments and data analysis, interpreted the results, performed a major part of the experiments and wrote the manuscript. OT and SGS prepared the plant extracts, participated in designing the study, and contributed to finalizing the manuscript.

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