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REGULATION OF CARBOHYDRATE METABOLISM AND INSULIN SECRETION IN DIABETIC RATS FOLLOWING TREATMENT WITH *Hypericum perforatum* L. HAIRY ROOT AQUEOUS METHANOL EXTRACT[1]

Elena Rafailovska[1], Oliver Tusevski[2], Sonja Gadzovska-Simic[2], Sasho Panov[3], Jasmina Petreska Stanoeva[4], Suzana Dinevska-Kjovkarovska[1], Biljana Miova[1*]

[1]*Department of Experimental Physiology and Biochemistry, Institute of Biology*
[2]*Laboratory of Plant Cell and Tissue Culture, Institute of Biology*
[3]*Department of Molecular Biology and Genetics, Institute of Biology*
[4]*Institute of Chemistry, Faculty of Natural Sciences and Mathematics,*
Ss. Cyril and Methodius University in Skopje
Skopje, R. North Macedonia

bmiova@pmf.ukim.mk

Hairy root (HR) cultures of *Hypericum perforatum* L. have shown promise in controlling hyperglycemia, regulating blood lipid and enzyme profiles, and improving metabolic function *in vivo*. These benefits are mainly attributed to the phenolic acids, flavonoids, and xanthones present in HR extracts. However, the specific mechanism underlying these effects remains unclear. This study was performed to elucidate the biochemical and molecular mechanisms driving HR the antihyperglycemic and antidiabetic effects of HR extracts.

HR extract (200 mg/kg body weight) was administered daily for 14 days to healthy rats and rats with streptozotocin-induced diabetes, with glibenclamide serving as a positive control. The phenolic composition of the HR extracts was confirmed through high-performance liquid chromatography/diode array detection/electrospray ionization mass spectrometry (HPLC/DAD/ESI-MS^n) analysis.

The results showed that HR extract treatment increased the plasma insulin level and pancreatic poly (adenosine diphosphate-ribose) polymerase (PARP) activity in diabetic rats, thus normalizing blood glucose levels. Additionally, it reduced the activity of gluconeogenic enzymes, increased the activity of glycolytic enzymes, and normalized the glycogen content in the liver. HR extract-treated rats also exhibited increased hepatic adenosine monophosphate-activated protein kinase (AMPK) mRNA expression and a decreased protein kinase Cε (PKCε) concentration.

In conclusion, HR extract demonstrated insulinotropic effects and effectively regulated hepatic carbohydrate metabolism in diabetic rats by modulating AMPK expression and the PKCε concentration. These findings suggest the potential use of HR extract as an herbal medicine for diabetes treatment and a source of antidiabetic drug development.

Keywords: xanthones; carbohydrate metabolism; adenosine monophosphate-activated protein kinase (AMPK); protein kinase Cε (PKCε); diabetic rats

РЕГУЛАЦИЈА НА ЈАГЛЕХИДРАТНИОТ МЕТАБОЛИЗАМ И СЕКРЕЦИЈАТА НА ИНСУЛИН КАЈ ДИЈАБЕТИЧНИ СТАОРЦИ ТРЕТИРАНИ НА МЕТАНОЛЕН ЕКСТРАКТ ОД ВЛАКНЕСТИ КОРЕНИ НА *Hypericum perforatum* L.

Културите од влакнести корени (HR) на *Hypericum perforatum* L. синтетизираат и акумулираат фенолни киселини, флавоноиди и ксантони, на чиешто присуство му се припишуваат досега познатите ефекти на екстрактите од HR кои се во насока на регулирање на

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хипергликемијата, липидниот и ензимски статус, како и метаболичките функции *in vivo*. Сепак, специфичниот механизам кој се однесува на овие ефекти не е целосно разјаснет.

Целта на ова истражување беше да се испитаат биохемиските и молекуларните механизми на антидијабетичното дејство на екстракт од влакнести корени (HR). Екстрактот од HR (200 mg/kg т.м.) беше аплициран еднаш дневно во текот на 14 дена кај здрави и стрептозотоцин-дијабетични животни. Како позитивна контрола беше користен третман со глибенкламид (Glb). Фенолните соединенија од екстракти од трансгените влакнести корени беа анализирани со методот HPLC/DAD/ESI-MS^n.

Добиените резултати покажаа дека екстрактот од HR значајно ја зголемува концентрацијата на инсулин во плазмата како и активноста на ензимот поли(ADP-рибоза) полимераза (PARP) во панкреасот кај дијабетични стаорци, што доведува до нормализирање на гликемијата. Покрај тоа, третманот со екстракт од HR ја намалува активноста на глуконеогенските ензими, ја зголемува активноста на гликолитичките ензими и го нормализира нивото на гликоген во црниот дроб. Дополнително, третманот со HR ја зголемува експресијата на протеин киназата активирана со AMP- (AMPK) и значајно ја намалува концентрацијата на протеин киназата Cε (PKCε) во црниот дроб.

Врз основа на добиените резултати може да се констатира дека екстрактот од HR поседува инсулинотропно дејство и го регулира јаглехидратниот метаболизам во црниот дроб кај дијабетичните стаорци преку модулација на експресијата на AMPK и концентрацијата на PKCε. Овие регулаторни ефекти го истакнуваат значењето на употребата на екстрактите од HR во третирање на состојбата на дијабет или како извор на компоненти со потенцијално антидијабетично дејство.

Клучни зборови: ксантони; јаглехидратен метаболизам; AMPK; PKCε; дијабетични стаорци

1. INTRODUCTION

Diabetes mellitus is a prevalent, chronic condition characterized by a reduced ability of the body to produce or respond to insulin, leading to hyperglycemia.[1] Finding cost-effective and low-risk ways to manage diabetes remains a significant challenge for healthcare systems. To address this challenge, natural low-cost phytochemicals with a low incidence of side effects offer a promising alternative treatment method for diabetes.[2]

Hairy root (HR) cultures are known for their potential to produce bioactive secondary metabolites at levels that are similar to or even higher than those found in wild-growing plants.[3] In particular, HR cultures obtained by *Agrobacterium rhizogenes*-mediated transformation have been proven to serve as a valuable experimental system for sustainable yield of secondary metabolites because of their rapid growth rate as well as their high genetic and biochemical stability.[4]

Agrobacterium rhizogenes-mediated transformation has been successfully established in many medicinal plants. Among these, *Hypericum perforatum* is the most popular medicinal plant and is characterized by antioxidant, antimicrobial, antiviral, anti-inflammatory, antitumor, hepatoprotective, and antidiabetic effects.[5] *Hypericum perforatum* HR cultures produce a variety of phenolic compounds, particularly xanthones, which accumulate in 2- to 4-fold higher amounts than in non-transformed roots.[6,7] Xanthones are distinguished by a wide range of biological activities (anticancer, anti-inflammatory, antimicrobial, antioxidant, and hepatoprotective), among which their antidiabetic activity is particularly strong.[8,9] In this context, the *in vitro* inhibitory activity of *H. perforatum* HR extracts against the enzymes α-amylase and α-glucosidase has been shown to be associated with the presence of mangiferin and γ-mangostin as the main representative prenylated xanthones.[10] In our previous study, we found that the HR aqueous methanol extract of *H. perforatum* is rich in xanthones and exhibits potent antihyperglycemic effects in diabetic rats by improving various metabolic parameters (*e.g.*, water and food consumption, urine production), blood lipids (triacylglycerols, cholesterol, and high-density lipoprotein), and serum enzymes (including aspartate aminotransferase, alanine transaminase, and gamma-glutamyl transferase).[11] However, further research is needed to elucidate the effects of HR extracts on carbohydrate metabolism and the molecular mechanisms underlying their antidiabetic action.

In diabetic conditions, hyperglycemia is primarily caused by the liver's production of glucose through gluconeogenesis and glycogenolysis.[12] Signaling pathways such as those involving adenosine monophosphate-activated protein kinase (AMPK) and protein kinase Cε (PKCε) regulate carbohydrate metabolism. AMPK serves as a key regulator of metabolism and suppresses gluconeogenesis,[13] whereas PKCε impairs the insulin signaling cascade and hepatic glucose production.[14] Studies have shown that xanthones might modulate the activity of carbohydrate metabolism-related

enzymes[15] and even affect the AMPK signaling pathway.[8] Based on the data mentioned above, we hypothesized that the antihyperglycemic effects *of H. perforatum* HR extracts are mediated by regulation of carbohydrate metabolism in the liver, potentially through modulation of the AMPK and PKCε signaling pathways.

The main aims of the present study were as follows:

(1) Identify and quantify various classes of phenolic compounds in HR extracts using high-performance liquid chromatography/diode array detection/electrospray ionization mass spectrometry (HPLC/DAD/ESI-MS^n).

(2) Assess the impact of HR extracts on the plasma insulin level and pancreatic poly(adenosine diphosphate-ribose) polymerase (PARP) activity, on hepatic key enzymes involved in carbohydrate metabolism, and on AMPK expression and the PKCε concentration in the liver of rats with streptozotocin (STZ)-induced diabetes.

2. EXPERIMENTAL SECTION

2.1. *Plant material and extract preparation*

In vitro-grown seedlings were established using seeds collected from wild-growing *H. perforatum* plants (voucher number 060231) taken from a natural population in the Republic of North Macedonia. The transformation of *H. perforatum* and establishment of HR cultures using *A. rhizogenes* A4 was previously described by Tuševski et al.[6] *Hypericum perforatum* HR was cultured in Murashige and Skoog liquid medium supplemented with Gamborg's B5 vitamins (MS/B_5 liquid medium) for 1 month and then collected and lyophilized. The dried material was subjected to phenolic compound extraction using 80 % aqueous methanol and an ultrasonic bath for 15 minutes. The resulting yellow methanolic extract was centrifuged at 13,000 rpm for 15 minutes and analyzed using HPLC/DAD/ESI-MS^n to identify and quantify the phenolic compounds.

2.2. *Identification and quantification of phenolic compounds by HPLC/DAD/ESI-MS^n*

The phenolic compounds were analyzed using an Agilent 1100 system (Agilent Technologies, Santa Clara, CA, USA) coupled with an ion trap mass detector. Chromatographic separation was carried out using an Agilent Zorbax Eclipse Plus C18 column (150 × 4.6 mm, 5 μm) with the following mobile phase composition: formic acid (1 %) as solvent A and methanol as solvent B. The gradient program was as follows: starting at 20 % B for 5 minutes (isocratic), increasing to 45 % B from 5 to 25 minutes, further increasing to 80 % B from 25 to 35 minutes, reaching 100 % B from 35 to 50 minutes, and maintaining 100 % B isocratically for the final 15 minutes. The temperature of the column, flow rate, and injection volume were set at 38 ºC, 0.4 ml/min, and 10 μl, respectively. Spectral data were accumulated in the range of 190 to 600 nm, and chromatograms were recorded at 260, 280, 330, and 350 nm.

For MS detection, negative ionization mode was used with nitrogen as the nebulizing gas at a pressure of 50 psi and a flow rate of 12 l/min. The capillary temperature and voltage were set at 350 ºC and 4 kV, respectively. MS spectra were collected from *m/z* 100 to 1200. Compound identification was conducted by comparing the ultraviolet (UV) and MS spectra with standard substances and literature data.[6,7]

Quantification was performed according to the area under the peaks in the UV chromatogram. Xanthones were quantified as mangiferin-equivalent at 260 nm, flavan-3-ols as epicatechin-equivalent at 280 nm, phenolic acids as caffeic acid-equivalent at 330 nm, and flavonol glycosides as quercetin-equivalent at 350 nm.

2.3. *Experimental animals*

In this study, 6- to 8-week old male Wistar rats (weight, 250 ± 20 g) were obtained from the Faculty of Natural Sciences and Mathematics in Skopje, R. North Macedonia and housed under standard conditions (temperature, 25 °C ± 2 °C; relative humidity, 55% ± 10%; 12-hour light/dark cycle). The rats were fed a standard pellet diet and had unlimited access to water. The study was approved by the Ethics Committee of the Faculty of Natural Sciences and Mathematics in Skopje, R. North Macedonia (03-2323/2) and conducted in accordance with EU Directive 2010/63/EU for the ethical treatment of laboratory animals.

2.4. *Preparation of HR extracts*

For the animal study, the extraction of phenolic compounds was performed by homogenization of 100 g powdered plant material with 0.5 l 80% (v/v) methanol/water at room temperature. The resulting extracts were centrifuged and dried using a rotary evaporator system (50 mbar, room temperature). The dried extract (extraction yield of 16% (m/m)) was then dissolved in 0.3 % carboxymethylcellulose (CMC) (w/v in distilled water).

A previously performed acute oral toxicity study showed that the extract was safe for consumption at a dose of 2000 mg/kg body weight.[11] Additionally, a previously performed dose-dependent study[11] led to the selection of 200 mg/kg body weight as the treatment dose for subsequent experiments.

2.5. *Induction of diabetes and experimental design*

The antihyperglycemic activity of the HR extracts was assessed in male rats with STZ-induced diabetes. Diabetes was induced using STZ at a dose of 45 mg/kg body weight, and the rats were considered diabetic if they had a fasting blood glucose level of >18 mmol/l after 7 days of STZ administration. The rats were divided into the following five groups of eight rats each: healthy rats treated with distilled water, healthy rats treated with 200 mg/kg body weight of HR extract (in 0.3 % CMC), diabetic rats treated with 0.3 % CMC, diabetic rats treated with 200 mg/kg body weight of HR extract (in 0.3 % CMC), and diabetic rats treated with 2.5 mg/kg body weight of glibenclamide (Glb) as a positive control. Treatments were administered intragastrically (*via* a feeding needle) once daily for 14 days after an 8-hour fast. After 14 days, the rats were sedated using sodium thiopental at 45 mg/kg body weight and then sacrificed using a normal laparotomy approach. Blood was taken from the dorsal vein and separated into serum and plasma tubes. Portions of the liver and pancreas were separated, washed with a cold saline solution, and promptly placed in liquid nitrogen (−196 °C) for preservation until analysis.

2.6. *Glucose and insulin measurements*

The serum glucose concentration was measured using a glucose oxidase/peroxidase-antiperoxidase (GOD-PAP) colorimetric kit (Human, Wiesbaden, Germany), and insulin levels were measured in plasma using an ultrasensitive rat insulin enzyme-linked immunosorbent assay kit (Mercodia, Uppsala, Sweden).

2.7. *Enzyme activity*

The pancreatic PARP activity was assessed using a Trevigen HT Universal PARP Assay Kit (Bio-Techne, Minneapolis, MN, USA) according to the manufacturer's protocol. The quantity of PKCε was measured in 5% liver homogenate using a PKCε enzyme-linked immunosorbent assay kit (USCN Life Science, Houston, TX, USA) in accordance with the manufacturer's protocol. The activity of the following key enzymes involved in glucose/glycogen metabolism were measured according to our previous study[16]: glucose-6-phosphatase (G6Pase), fructose-1,6-bisphosphatase (F16BPase), glycogen phosphorylase (GPase), hexokinase (HK), and glucose-6-phosphatase dehydrogenase (G6PDH). The liver glycogen, glucose, and glucose-6-phosphate (G6P) content was also measured.

2.8. *Relative gene expression*

RNA was extracted from liver tissue samples using the Gene JET RNA kit for mRNA isolation (Thermo Scientific, Waltham, MA, USA). The amount of RNA was then measured using a Qubit 4 Fluorometer (Invitrogen/Thermo Fisher Scientific). The extracted RNA was converted into complementary DNA using a TaqMan reverse transcription reagent kit (Life Technologies/Thermo Fisher Scientific). Real-time quantification was performed on a StepOne Real-Time Polymerase Chain Reaction System (Applied Biosystems/Thermo Fisher Scientific) using SYBR Green Master Mix (Thermo Fisher Scientific). The housekeeping gene β-actin was used as an internal control for the experiment. The primers used for AMPKα1 were as described previously.[17] The data were analyzed using the comparative cycle threshold approach.

2.9. *Statistical analysis*

The results are shown as average values with standard deviation. Analyses were performed with one-way analysis of variance and Tukey's post-hoc test using GraphPad Prism 9 (GraphPad Software, La Jolla, CA, USA). Results were considered statistically significant at a p-value of < 0.05.

3. RESULTS AND DISCUSSION

3.1. *Identification and quantification of phenolic compounds*

The data concerning the identification and quantification of phenolic compounds in the *H. perforatum* HR extracts are summarized in Table 1. The chemical structures of the compounds are presented in Figure 1. The HPLC-DAD chromatograms of *H. perforatum* HR extracts at 260 and 280 nm are shown in Figure 2. In brief, five groups of phenolic compounds were recorded in the plant extracts: phenolic acids, flavan-3-ols, flavonol glycosides, dihydrochalcones, and xanthones. Their identification was based on the UV/visible spectral data and LC/MS in the negative $[M–H]^−$ ionization mode with subsequent MS^2 and MS^3 analysis for further identification with reference to similar previously reported data.[6]

T a b l e 1

*Identification and quantification of phenolic compounds in Hypericum perforatum hairy root extracts**

Compounds		t_R (min)	UV (nm)	[M–H]⁻	MS²	Contents (mg/100 g DW)
Phenolic acids						
F1	Quinic acid	4	262, 310	191	173, **127**	19.22
F2	3-Cafeoylquinic acid	6.2	240, 294sh, 326	353	**191**, 179, 135	4.69
F3	5-Cafeoylquinic acid[a]	6.8	240, 294sh, 326	353	191, **179**, 135	5.98
F4	3-p-Coumaroylquinic acid	23.5	314	337	191, **163**	6.12
	Total					**36.01**
Flavan-3-ols						
F5	Procyanidin dimer B type	24.1	280	577	559, 451, **425**, 407, 289	11.90
F6	Epicatechin	26.2	280	289	245, 205	4.20
F7	Procyanidin dimer B type	28.1	280	577	559, 451, **425**, 407, 289	26.15
F10	Procyanidin dimer B type	34.2	280	577	559, 451, **425**, 407, 289	95.41
	Total					**137.67**
Flavonol glycosides						
F9	Quercetin 6-C-glucoside	33.2	256, 356	421	331, **301**	37.83
F11	Kaempferol hexoside	36.3	256, 266, 350	447	285	24.85
	Total					**62.67**
Dihydrochalcones						
F8	Nothofagin (Phloretin-C-hexoside)	32.8	280	435	**315**, 287, 271	**44.58**
Xanthones						
X1	Mangiferin[a]	30.8	238, 256, 312, 362	421	331, 301, **258**	26.99
X2	Xanthone derivative 1	31.6	264, 294	589	**473**, 421, 357, 305	30.80
X3	Mangiferin isomer	34.9	260, 318, 382sh	421	331, **301**	97.83
X4	Trihydroxyxanthone-sulfonate	35.6	222, 314sh	323	243	56.91
X5	Tetrahydroxyxanthone-C-pentoside	37.9	216, 252, 284sh, 326	391	331, **301**	2.31
X6	Xanthone derivative 2	39.4	242, 306	367	**287**	10.98
X7	1,3,5,6-Tetrahydroxyxanthone	40.5	250, 282, 328	259	**229**, 213, 187	9.20
X8	1,3,6,7-Tetrahydroxyxanthone	42.0	236, 254, 314, 364	259	231, **215**, 187, 147	5.98
X9	Xanthone derivative 3	43.8	244, 280, 316	353	**273**	10.29
X10	1,3,6,7-Tetrahydroxyxanthone 8-prenyl xanthone	45.8	248, 312, 366	327	325, **297**, 258,201	1.36
X11	1,3,5,6-Tetrahydroxyxanthone 8-prenyl xanthone	47.1	242, 260, 320, 368	327	325, **297**, 258, 201	11.40
X12	1,3,7-Trihydroxy-2-(2-hydroxy-3-methyl-3-butenyl)-xanthone	48.9	238, 260, 314, 388	327	**309**, 257	6.53
X13	Paxanthone	50.5	244, 264, 324, 386	339	**324**, 307	4.00
X14	γ-Mangostin isomer	51.4	254, 286, 324	395	326, 283, **271**	19.20
X15	γ-Mangostin isomer	52.0	260, 316, 370	395	351, **339**, 326, 283	21.12
X16	Trihydroxy-1-metoxy-C-prenyl xanthone	52.3	260, 286, 314	341	**325**	9.70
X17	Garcinone E	53.1	256, 286, 332	463	394, 351, **339**, 297, 285	76.28
X18	α-Mangostin	53.4	254, 330	409	394, **351**, 325, 272	3.66
X19	Xanthone derivative 4	55.6	270, 330, 400	467	398, 383, 327, 271, 234	13.01
X20	Xanthone derivative 5	56.1	254, 284, 326	481	**412**, 397, 327, 271, 234	7.67
X21	Xanthone derivative 6	57.6	256, 282, 326sh	535	**466**, 383, 315	9.29
	Total					**499.27**
	Total phenolic compounds					**735.62**

*MS² ions in bold indicate the base peak. DW: dry weight, sh: shoulder, t_R: retention time.

[a]Compounds identified with authentic standards.

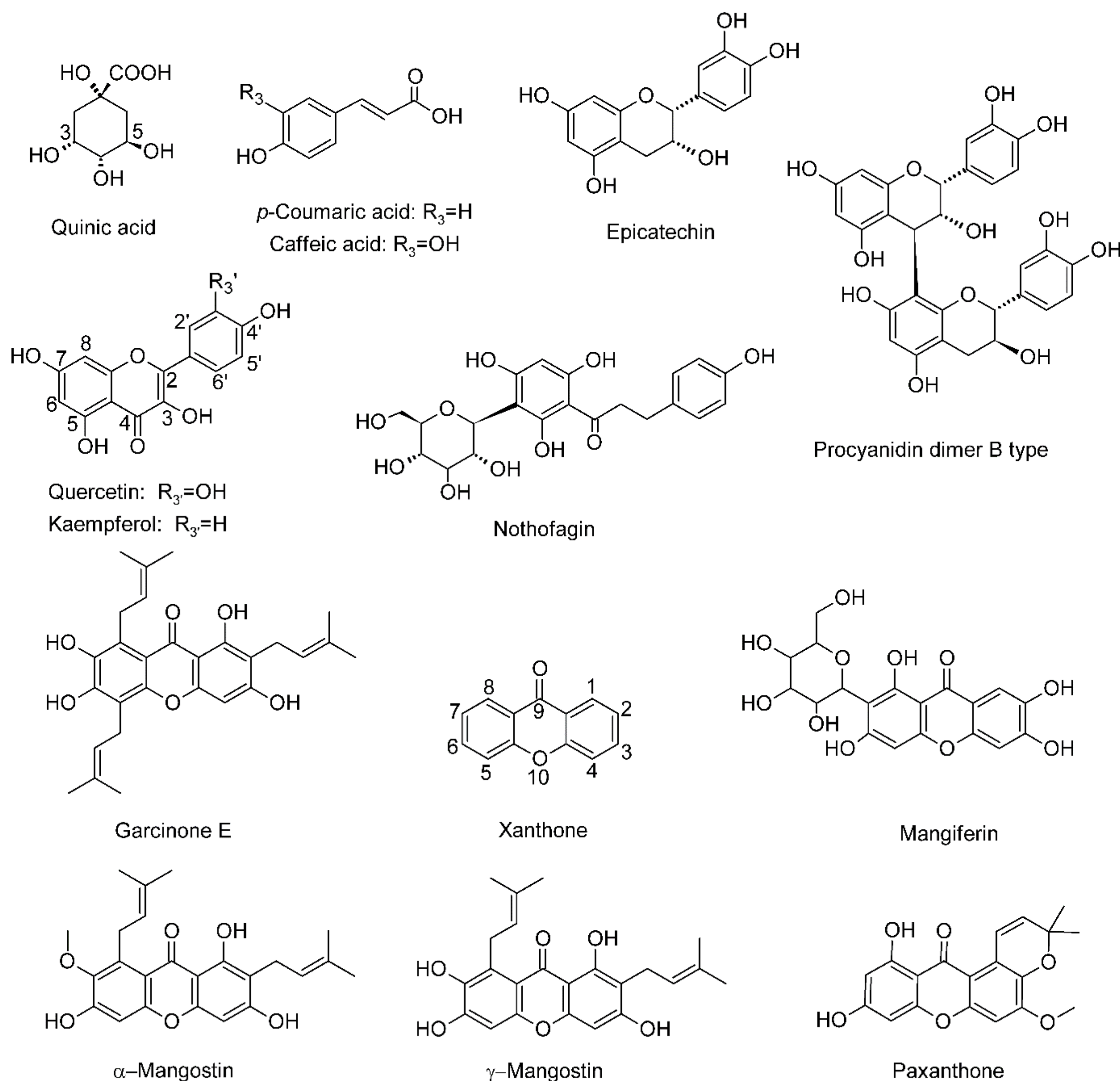

Fig. 1. Chemical structures of phenolic acids, flavonoids, and xanthones detected in *Hypericum perforatum* HR extracts

The chromatographic analysis of *H. perforatum* HR extract revealed that xanthones represented the dominant class among all phenolic compounds, which is consistent with our previous results.[6,7] Five groups of phenolic compounds were characterized in the plant extracts: phenolic acids, flavan-3-ols, flavonol glycosides, dihydrochalcones, and xanthones. Among the identified compounds, mangiferin, γ-mangostin isomers, and trihydroxy-1-methoxy-*C*-prenylxanthone were found to be dominant xanthones in the *H. perforatum* HR extracts. Xanthones are structurally diverse secondary metabolites with strong antidiabetic activity.[10,11,22] Our previous *in vitro* and *in silico* investigations revealed that xanthones greatly contribute to the antidiabetic activity of *H. perforatum* HR extracts.[10] In support of these results, we discovered that *H. perforatum* HR extract effectively regulates blood glucose and various metabolic parameters in rats with STZ-induced diabetes.[11] Therefore, in the present study, we investigated the mechanism underlying the antihyperglycemic and antidiabetic effects of *H. perforatum* HR extract in rats with STZ-induced diabetes.

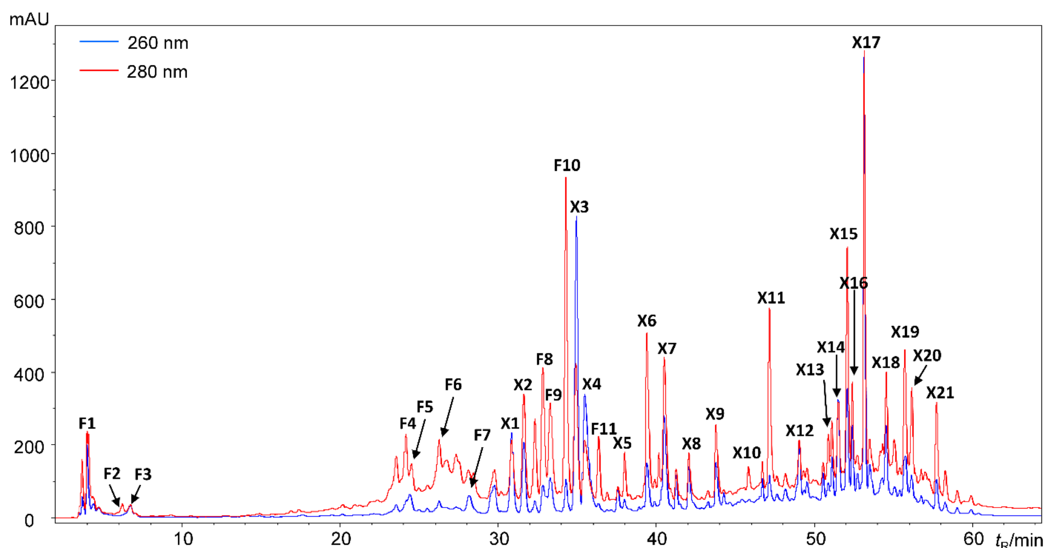

Fig. 2. HPLC-DAD chromatograms of *Hypericum perforatum* HR extracts at 260 nm (in blue) and at 280 nm (in red). For peak identity see Table 1.

3.2. *Blood glucose concentration, plasma insulin concentration, and pancreatic PARP activity*

Figure 3 shows the data regarding the blood glucose concentration, plasma insulin concentration, and pancreatic PARP activity. The diabetic rats demonstrated a 3-fold elevation in the blood glucose concentration relative to their healthy counterparts, alongside a notable reduction in the plasma insulin concentration (−57%, $p < 0.05$) and a significant decrease in PARP activity (−53%, $p < 0.05$). Upon administration of HR extract to the diabetic rats, there was a considerable reduction in the blood glucose concentration, effectively lowering it to the control level.

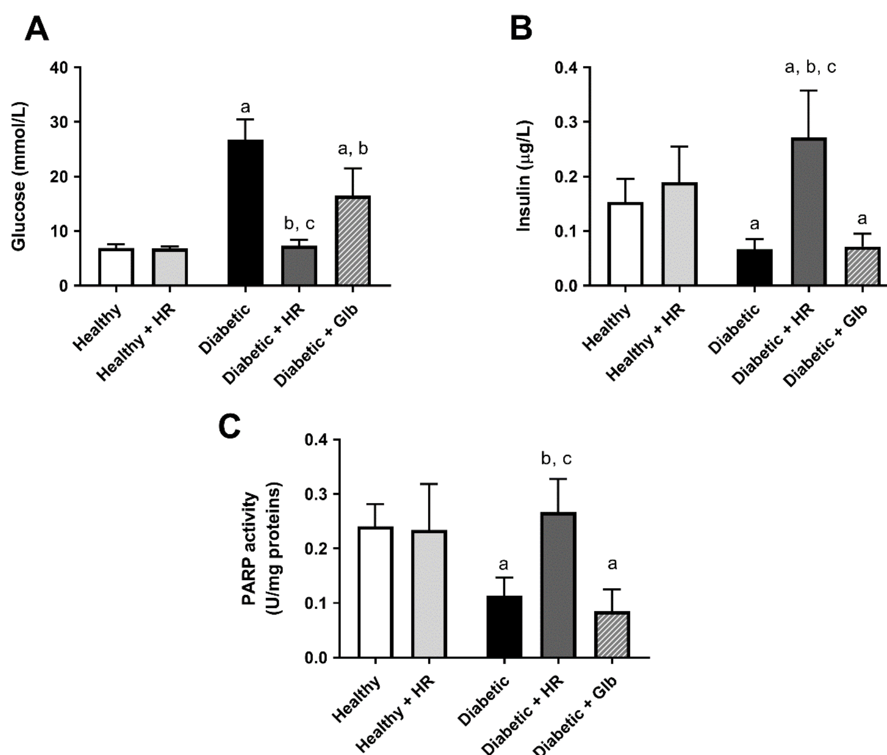

Fig. 3. (A) Blood glucose concentration, (B) plasma insulin concentration, and (C) pancreatic PARP activity in healthy and diabetic rats treated with HR extracts and Glb. Significant differences ($p < 0.05$): [a]compared to healthy rats, [b]compared to diabetic rats, [c]compared to diabetic treated with Glb

Moreover, HR extract treatment led to a 3-fold increase in the insulin concentration ($p < 0.05$) and a significant elevation in pancreatic PARP activity relative to the diabetic controls. STZ is β-cytotoxic agent that promotes reactive oxygen species-induced DNA damage in β-cells, causing PARP hyperactivation and fragmentation,[18,19] and it decreases PARP activity *via* loss of β-cells.[20] Considering that the restoration of PARP activity correlates with DNA synthesis in normal cellular proliferation,[21] the obtained results suggest that the HR extract might exert a cytoprotective and regenerative effect on the β-cells of diabetic rats.

Recent research has indicated that prenylated xanthones, such as those found in HR extract (α-mangostin, γ-mangostin, and garcinone E), can serve as novel drugs for antidiabetic therapy by regeneration of β-cells.[22] Mangiferin also promotes islet regeneration and enhances β-cell proliferation[23] through decreased PARP cleavage.[24] Although HR extract treatment exhibited similar effects to Glb regarding insulin levels and PARP activity, it was notably more effective in controlling blood glucose levels.

3.3. *Carbohydrate metabolism in the liver*

Regarding liver carbohydrate metabolism (Figs. 4 and 5), we found a significant 2-fold increase in the hepatic glucose concentration ($p < 0.05$) alongside a 50 % decrease in G6P content ($p < 0.05$) (Fig. 4A, B). Moreover, the gluconeogenic enzymes G6Pase (Fig. 4C) and F16BPase (Fig. 4D) were significantly increased in diabetic rats (56 % and 88 %, respectively). Additionally, diabetic conditions led to a substantial 78% reduction in hepatic glycogen content (Fig. 5D) ($p < 0.05$) and a decrease in the activity of hepatic GPase (Fig. 5C) by 46 %, HK (Fig. 5A) by 67 %, and G6PDH (Fig. 5B) by 56 % (all $p < 0.05$) compared to control rats.

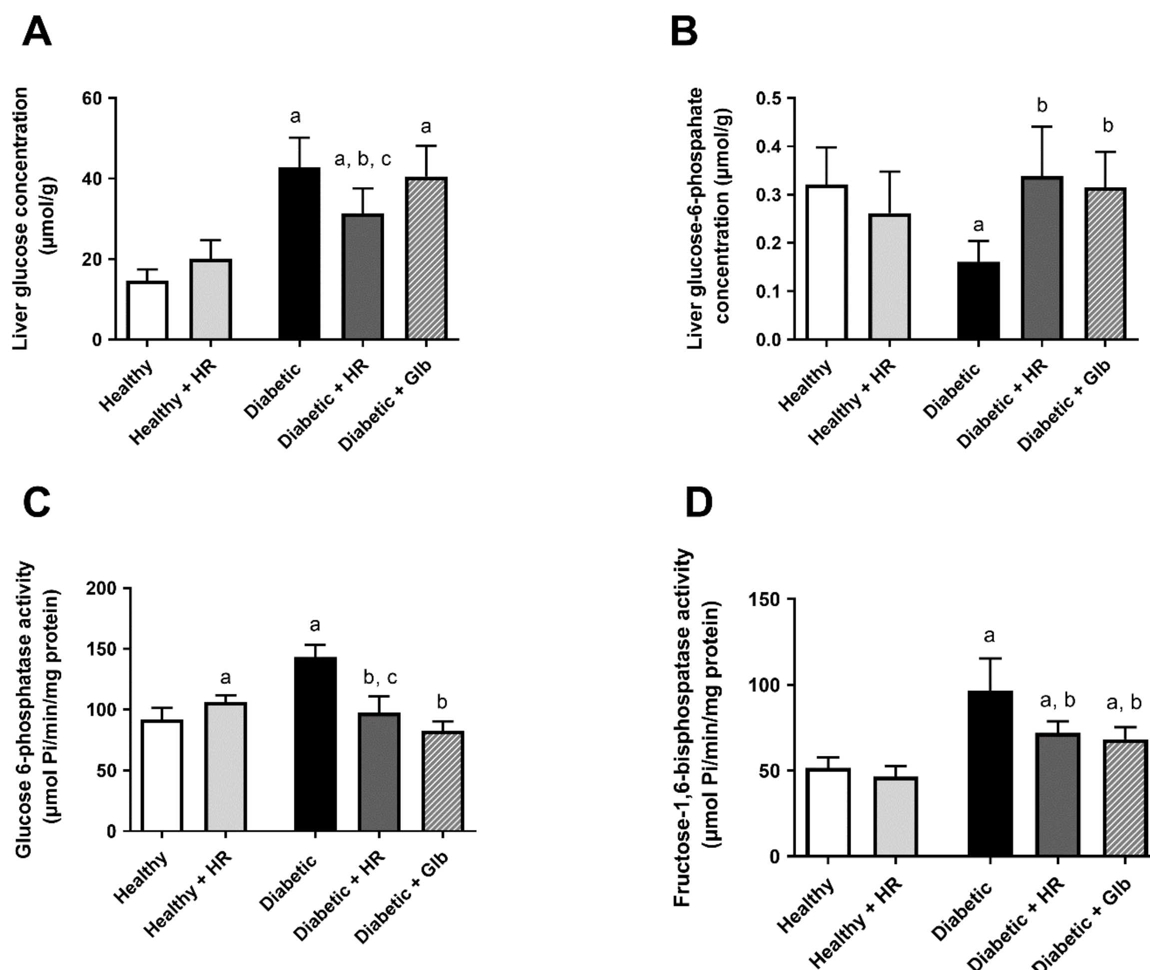

Fig. 4. Hepatic (A) glucose concentration, (B) G6P concentration, (C) G6Pase activity, and (D) F16BPase activity in healthy and diabetic rats treated with HR extracts and Glb. Significant differences ($p < 0.05$): [a]compared to healthy rats, [b]compared to diabetic rats, [c]compared to diabetic treated with Glb

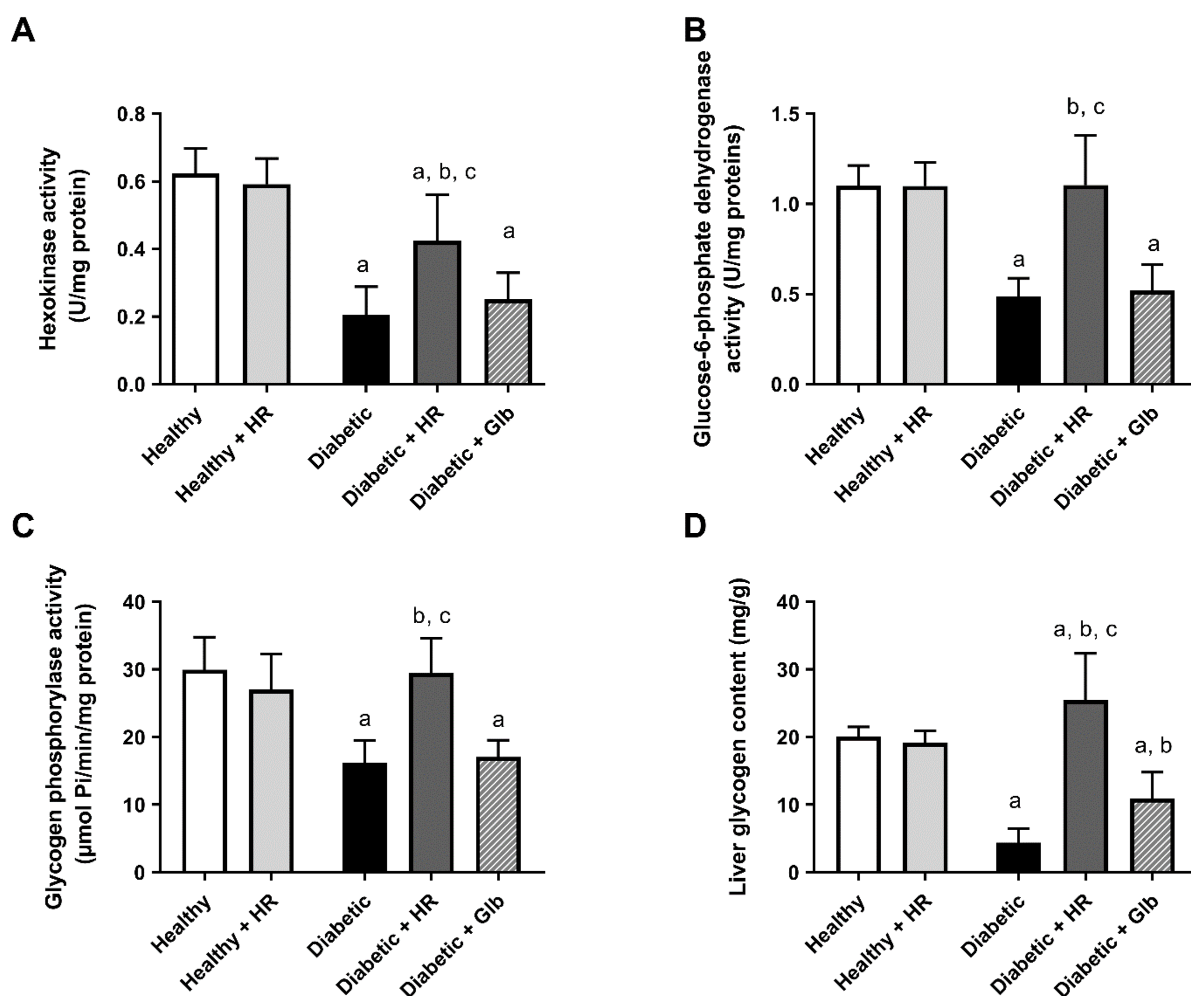

Fig. 5. Hepatic (A) HK, (B) G6PDH, and (C) G6Pase activity, and (D) glycogen content in healthy and diabetic rats treated with HR extracts and Glb. Significant differences ($p < 0.05$): [a]compared to healthy rats, [b]compared to diabetic rats, [c]compared to diabetic treated with Glb.

Thus, experimental diabetes increased gluconeogenesis and decreased hepatic glycogen, glycolysis, and pentose–phosphate pathway activity, which is consistent with previous research.[16] These changes were accompanied by a significant decrease in the hepatic AMPKα mRNA level (56 %, $p < 0.05$) and a considerable increase in the PKCε concentration (71 %, $p < 0.05$) relative to control rats (Fig. 5).

Decreased AMPK-α1 activity in diabetic conditions has been associated with reduced activation of the AMPK signaling pathway,[25] which further inhibits glycolysis and glycogen production and stimulates hepatic gluconeogenesis,[26] as shown in the present study. Elevated levels of intracellular glucose, also shown the present study, trigger PKCε activation, which subsequently suppresses the AMPK signaling pathway due to direct phosphorylation by PKCε.[27]

This study revealed that HR extracts reversed diabetic-induced alterations in carbohydrate metabolism in the liver. Figure 4 shows that relative to diabetic animals, HR extract treatment resulted in a significant reduction in the hepatic glucose concentration by 28 % ($p < 0.05$) (Fig. 4A), a marked elevation in the G6P concentration by 110 % ($p < 0.05$) (Fig. 4B), and normalization of values to those of the control group. Additionally, HR extract treatment significantly decreased the activity of G6Pase (Fig. 4C) and F16BPase (Fig. 4D) by 32 % and 26 %, respectively, demonstrating a trend toward the values observed in the control group. This ultimately resulted in normalization of hyperglycemia in this group of rats. Previous research demonstrated that flavonol glycosides, which were confirmed in the present HR extract, decrease glucose production in hepatocytes by suppressing G6Pase activity.[25] Xanthones such as α-mangostin and mangiferin reduce the activity of G6Pase and F16BPase.[29]

Therefore, we consider that the inhibition of gluconeogenesis by HR extracts could be associated with the presence of flavonol glycosides and xanthones.

The HR extract treatment effectively restored liver glycogen metabolism by significantly increasing hepatic glycogen levels (approximately 5-fold, $p < 0.05$), surpassing control values (Fig. 5D). Additionally, HR extract treatment notably enhanced the activity of GPase by 81% ($p < 0.05$) (Fig. 5C), HK by 107% ($p < 0.05$) (Fig. 5A), and G6PDH by 128% ($p < 0.05$) (Fig. 5B), thereby tending to normalize these parameters toward control levels. We confirmed the occurrence of insulin-stimulated glycogen synthesis[28] in diabetic rats treated with HR extract after observing a 3-fold increase in the insulin concentration. Mangiferin has been shown to induce enzymes involved in glycogen synthesis.[15] In addition, we found a significant improvement in the activity of HK and G6PDH, which was followed by a decrease in the hepatic glucose level and an increase in the hepatic G6P level, indicating increased rates of glycolysis and glucose utilization by the liver via the pentose–phosphate cycle in the liver of HR extract-treated rats. Published data show that α-mangostin and mangiferin increase HK activity in diabetic rats.[29] However, whether this increase in glycolytic enzymatic activity is a result of HR-mediated insulin release or the insulin-mimetic effects of the components from the HR extract remains unclear.

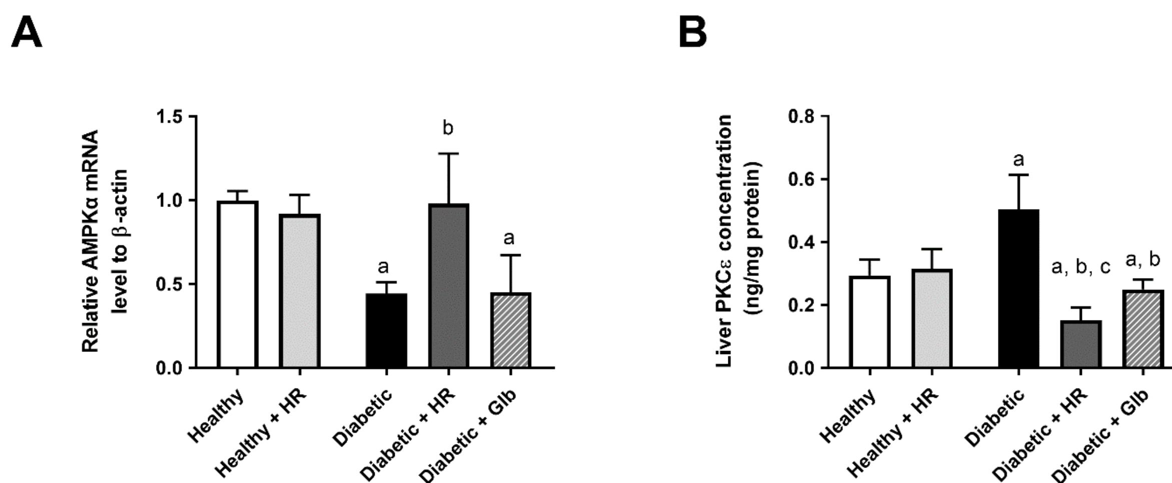

Fig. 6. Hepatic (A) AMPKα mRNA level and (B) PKCε concentration in healthy and diabetic rats treated with HR extracts and Glb. Significant differences ($p < 0.05$): [a]compared to healthy rats, [b]compared to diabetic rats, [c]compared to diabetic rats treated with Glb

We further investigated the exact molecular mechanism by which HR extract affects carbohydrate metabolism in the liver. We found that HR extract treatment caused an increase in AMPK mRNA levels up to control values (121%, $p < 0.05$) (Fig. 6A).

Presumably, the reduced activity of gluconeogenic enzymes (G6Pase and F16BPase) in the liver of HR extract-treated animals is due to activation of the AMPK signaling pathway. This is based on the fact that AMPK also reduces the gene expression of G6Pase and phosphoenolpyruvate carboxykinase.[30] According to Ruderman and Prentki,[30] activated AMPK also induces genes involved in glycogen metabolism, such as the *Pygl* gene, encoding the enzyme GPase. This might explain the pronounced increase in the liver glycogen content in HR extract-treated animals. Various studies have shown that xanthones such as γ-mangostin,[31] α-mangostin,[32] and mangiferin[33] activate the AMPK signaling pathway through either increasing the AMP/ATP ratio or elevating AMPK expression. Moreover, recent research indicates that quercetin, particularly its glycosides, enhances glucose uptake through AMPK stimulation.[34,35] Considering these data, it is likely that the xanthones and quercetin 6-C-glucoside present in HR extract contribute to the increased AMPK expression observed in diabetic rats treated with HR extract.

In the present study, HR extract treatment also reduced the concentration of PKCε (−70%, $p < 0.05$) in diabetic animals (Fig. 6B). This likely enhanced insulin signaling in the liver as evidenced by the increase in glycogen content, increase in glycolytic enzyme activity, and decrease in gluconeogenic enzyme activity in HR extract-treated rats. These results align with previous studies of the impact of xanthones on the PKCε enzyme.[34] Specifically, mangiferin modulates the activity of several PKC isoforms (PKCα, PKCβ, and PKCε) through its interaction

with signaling molecules and transcription factors.[36] The authors of these studies found a decrease in the expression of all PKC isoforms in the renal tissue of rats with STZ-induced diabetes after 30 days of mangiferin treatment.

This study represents the first step toward a more profound understanding of the antidiabetic effects of xanthone-rich extract from *H. perforatum* HR cultures.

4. CONCLUSION

This comprehensive HPLC/DAD/ESI-MS^n analysis of *H. perforatum* HR extracts revealed a rich chemical composition of phenolic compounds. In total, 32 compounds were detected and quantified, with the highest diversity and content found in xanthone derivatives (68 % of the total phenolic content), followed by flavan-3-ols (18 % of the total phenolic content). The content of phenolic acids, flavonols, and dihydrochalcones was quite similar, each accounting for approximately 5% of the total phenolic content.

The xanthone-rich HR extract (80% (v/v) methanol/water) of *H. perforatum* normalizes hyperglycemia, increases insulin levels, and normalizes PARP activity in the pancreas of diabetic rats. HR extract treatment of diabetic rats also reduces hepatic gluconeogenesis, restores normal glycogen metabolism, and increases glucose utilization through the glycolysis and pentose–phosphate pathway. The HR extract exhibits this action in the liver by inhibiting PKC and activating the AMPK signaling pathway. Along with its previously demonstrated glucose-lowering effect *in vivo*, we propose that *H. perforatum* HR extract can serve as a potent herbal medicine for treating diabetes mellitus and a promising source of compounds for antidiabetic drug development.

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