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HYPOGLYCEMIC ACTIVITY OF GLYCYRRHIZIC ACID AND SOME OF ITS DERIVATIVES IN THE ALLOXAN DIABETES MODEL IN RATS

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The hypoglycemic activity of glycyrrhizic acid (GA, **I**), its trisodium (**II**) and sodium-dilithium salts (**III**), and a conjugate with *L*-methionine methyl ester (**IV**) was studied using an alloxan diabetes model after peroral administration to male Wistar rats. It was found that GA and its conjugate **IV** at a dose of 100 mg/kg exhibited hypoglycemic activity and reduced the blood glucose content of the animals by 35.5 and 42.6%, respectively, after 120 min. GA conjugate **IV** had low toxicity and hypoglycemic activity superior to those of GA and acarbose.

Keywords: glycyrrhizic acid, conjugate, *L*-methionine methyl ester, hypoglycemic activity, diabetes mellitus, alloxan, rats.

Diabetes mellitus (DM) is one of the most prevalent metabolic diseases related to disturbed metabolism of carbohydrates, proteins, fats, water, and electrolytes [1, 2]. DM is divided into two types, i.e., *type 1* and *type 2*, based on the pathogenic processes leading to hyperglycemia. *Type 1 DM* (DM1, autoimmune diabetes) develops as the result of immune destruction of pancreatic β-cells, which leads to an insulin deficit, hyperglycemia, and complications [1]. *Type 2 DM* (DM2, insulin-independent diabetes) is related to disturbed glycogen metabolism in the liver, disturbed glucose homeostasis, a deficit of insulin secretion, or resistance to insulin [2, 3]. All currently existing antidiabetic drugs possess multiple side effects (toxicity, nausea, diarrhea, weight gain, high resistance, etc.) that limit their use in medicine [3 – 6]. The broad incidence and duration of the disease and the various complications (nephropathy, neuropathy, retinopathy, cataracts, etc.) associated with diabetes and the development of resistance to insulin make the search for antidiabetic agents critical.

A promising approach of modern medicinal chemistry and pharmacy is to seek new antidiabetic agents among natural compounds of plant origin (plant secondary metabolites) and to use available secondary metabolites with proven antidiabetic activity as platforms to construct new medicines for treating diabetes and other metabolic diseases [7 – 9]. Saponins from several medicinal plants are potential antidiabetic agents [10].

Glycyrrhizic acid (GA, **I**) is the major triterpene saponin in the extract of licorice roots (*Glycyrrhiza glabra* and *G. uralensis*) (Leguminosae) and possesses a broad spectrum of biological activity (anti-inflammatory, antiulcer, antioxidant, antiallergic, antiviral, antitumor, antiallergic, heptoprotective, etc.) [11, 12]. GA is a regulator of metabolic syndrome associated with obesity and insulin-resistance, exhibits hypolipidemic and antidiabetic activity, and inhibits 11β-hydroxysteroid dehydrogenases (11β-HSD) that are responsible for glucocorticoid metabolism [11, 13]. Development of 11β-HSD is considered a promising strategy for discovering new medicines for treating type 2 DM and obesity [14 – 16]. GA was shown to significantly decrease hyperglycemia and

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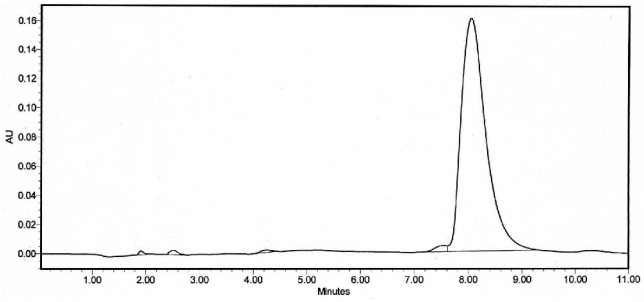
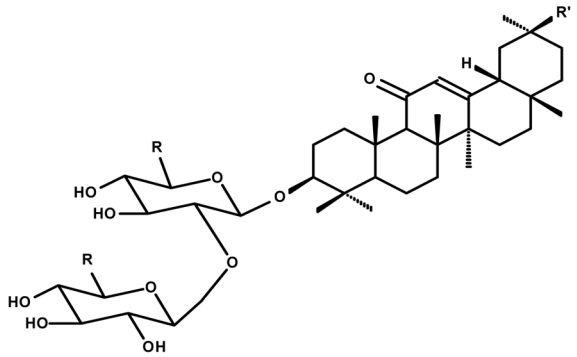


Fig. 1. HPLC chromatogram of glycyrrhizic acid (**I**). Atlantis C18 column (3.9 × 300 mm), mobile phase (MP) - MeOH–HOAc (0.3 N), 80:20 (vol%); MP flow rate, 1 mL/min; spectrophotometric diode-array detector at λ = 254 nm. Retention time (RT), 8.04 min; content of main compound, 96.1 ± 0.5%.



I: R = R′ = OH;

II: R = COOH, R′ = $COO–Na^+$;

III: R = $COO–Li+$, R′ = $COO–Na^+$;

IV: R = $CH_3SCH_2CH_2CH(NHCO)COOCH_3$, R′ = COOH

hyperlipidemia and oxidative stress associated with them and to alleviate pancreatic and renal anomalies caused by diabetes [12]. GA upon peroral administration at a dose of 100 mg/kg over 7 d to rats significantly reduced hyperglycemia by increasing the sensitivity to insulin and regulating glucose metabolism [17]. The ability of GA to reduce the postprandial blood glucose level in mice for an insulin-independent model and after feeding for seven weeks was also reported [18]. GA reduced hyperglycemia in rats with a DM1 model induced by streptozotocin [19]. GA of purity 93.5% inhibited *in vitro* α-glucosidase activity with IC_{50} of 1.88 mM, analogously to the known antidiabetic drug acarbose and reduced the blood glucose level in mice with DM2 [20]. GA also enhanced secretion of glucagon-like peptide-1 (GLP-1), which is broadly employed in therapy of DM1, by activating Takeda G-protein-coupled receptor 5 (TGR5) in rats with streptozotocin diabetes [21]. The ability of GA to reduce the blood glucose level *in vivo* with a low insulin content indicates that the licorice root glycoside is promising as a preventative and therapeutic agent for controlling insulin-independent DM2 and as a platform for producing new antidiabetic agents. The hypoglycemic activity of GA and its derivatives in the alloxan diabetes model *in vivo* has not been reported.

The present work reports the hypoglycemic activity of GA of purity 96% obtained by us from the commercially available monoammonium salt (glycyram) [22], its trisodium (**II**) [23] and sodium-dilithium salts (**III**) [24], and a conjugate of GA with *L*-methionine methyl ester that was synthesized for the first time in an alloxan diabetes model in rats. Alloxan causes a mixed form of DM because it leads to reduced functioning of β-cells and their partial death [25].

EXPERIMENTAL CHEMICAL PART

Conjugate **IV** was synthesized by condensing GA with *L*-methionine methyl ester in a dioxane–DMF mixture using *N*-hydroxysuccinimide and *N*,*N*′-dicyclohexylcarbodiimide in the presence of Et_3N at 20 – 22°C for 24 h. Target product **IV** was isolated in 56% yield by column chromatography over silica gel. The structure of **IV** was confirmed by IR and NMR spectral data. The purity of the synthesized compounds was monitored by HPLC and was 95 – 97% (Figs. 1 – 4).

PMR and ^{13}C NMR spectra were recorded with TMS internal standard on a Bruker AMX-300 spectrometer at operating frequency 300 (^1H) and 75.5 MHz (^{13}C). IR spectra were taken from Vaseline oil mulls on a Prestige-21 FTIR spectrophotometer (Shimadzu). Optical activity was measured in a 1-dm tube at 20 – 22°C (λ_{Na} 546 nm) on a PerkinElmer 241 polarimeter.

TLC used Sorbfil plates (Sorbpolimer, Russia). Spots of compounds were detected by H_2SO_4 (5%) in EtOH followed by heating at 120 – 130°C for 2 – 3 min. Column chromatography used silica gel (50 – 160 μm fraction, IMID LLC, Russia). HPLC analysis of GA was performed on a Shimadzu LC-20 liquid chromatograph (Japan) over Atlantis C18 (3.9 × 300 mm) and Vydac C18 (4.6 × 250 mm) reversed-phase columns using a mobile phase of MeOH–HOAc (0.3 N), 80:20 (vol%) at flow rate 1 mL/min and a spectrophotometric diode-array detector at λ = 254 nm.

GA with a content of main compound of 96.1% (HPLC) (Fig. 1) that was obtained as before [22] from commercially available glycyram, *N*-hydroxysuccinimide, and *N*,*N*′-dicyclohexylcarbodiimide (Sigma-Aldrich, USA) and *L*-methionine methyl ester hydrochloride (Reanal, Hungary) were used in the work. The trisodium salt of GA (**II**) was prepared according to the literature [23] in 97.5% purity (Fig. 2); the sodium-dilithium salt (**III**), as before [24] (94.7% purity) (Fig. 3). The physicochemical properties of GA salts **II** and **III** agreed with the literature data. The elemental analyses agreed with those calculated. Solvents were distilled beforehand and purified by standard methods [25].

3-*O*-{2-*O*-[*N*-(β-*D*-Glucopyranosyluronoyl)-*L*-methionine methyl ester]-*N*-(β-*D*-glucopyranosyluronoyl)-*L*-methionine methyl ester}-(3β,20β)-11-oxo-30-norolean-12-

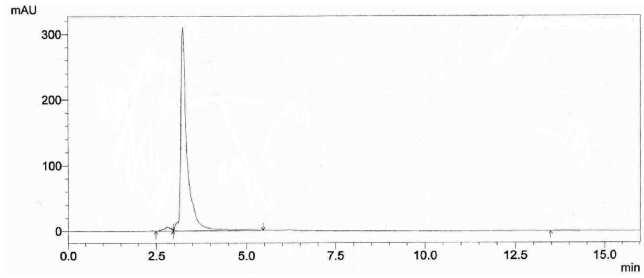


Fig. 2. HPLC chromatogram of trisodium salt of GA (**II**). Vydac C18 column (4.6 × 250 nm); mobile phase MeOH–HOAc (0.3 N), 80:20 (vol%); flow rate, 1 mL/min; spectrophotometric diode-array detector at λ = 254 nm. RT = 3.23 min; content of main compound 97.5 ± 0.5%.

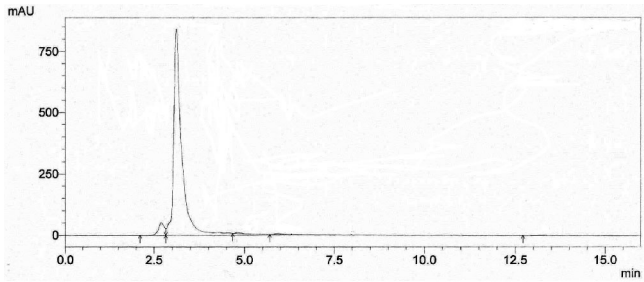


Fig. 3. HPLC chromatogram of sodium-dilithium salt of GA (**III**). Vydac C18 column (4.6 × 250 mm); mobile phase MeOH–HOAc (0.3 N), 80:20 (vol%); flow rate, 1 mL/min; spectrophotometric diode-array detector at λ = 254 nm. RT = 3.11 min; content of main compound 94.7 ± 0.5%.

ene (IV). A solution of GA (1.64 g, 2 mmol) in dioxane (50 mL) was treated with *N*-hydroxysuccinimide (1.15 g, 10.0 mmol) and *N*,*N*′-dicyclohexylcarbodiimide (1.24 g, 6 mmol). The mixture was stirred at room temperature (20 – 22°C) for 5 h. The precipitate of dicyclohexylurea was filtered off. The filtrate was treated with *L*-methionine methyl ester (0.98 g, 6.0 mmol), DMF (5 mL), and Et_3N (1.2 mL). The mixture was stored at room temperature for 24 h with periodic stirring, diluted with cold H_2O, and acidified with citric acid (pH ~ 4). The precipitate was filtered off, rinsed with H_2O, dried, and chromatographed over a column of silica gel with elution by $CHCl_3$–EtOH mixtures (300:10, 200:10, 100:10, 50:10, vol%) with TLC monitoring. Yield 56% (amorphous powder), 96.2% purity according to HPLC (Fig. 4). R_f 0.52 ($CHCl_3$–EtOH, 5:1). $[\alpha]_D^{20}$ + 56° (c 0.02, MeOH). both s(ν, cm^{-1}): 3500 – 3200 (OH, NH); 1739 (COOH); 1656 (C11=O); 1534 (CONH). PMR spectrum (CD_3OD, δ, ppm): 5.56 (1H, s, H12); 4.64 – 4.50 (2H, m, H-1″, H-1′); 4.22 – 4.12 (5H, m, H2″, H3″, H3′, H2′, H3); 3.76 (2H, s, H4″, H4′); 3.69, 3.66 (6H, both s, $2OCH_3$); 3.61 – 3.58 (2H, m, H5″, H5′); 3.50 – 3.00 (7H, m, CH, CH_2); 2.92 – 2.42 (16H, m); 2.18 – 1.60 (12H, m); 1.40 (3H, s, CH_3); 1.28 (3H, s, CH_3), 1.23 (6H, s, $2CH_3$), 1.18 – 1.16 (3H, m); 1.14 (3H, s, CH_3); 1.12 (3H, s, CH_3); 1.05 – 0.89 (5H, m, CH, CH_2); 0.81 (3H, s, CH_3); 0.79 – 0.75 (2H, m, 2CH). ^{13}C NMR spectrum (CD_3OD, δ, ppm): 202.7 (C11), 179.2 (C30), 172.9, 171.7, 171.5 (C13, C6″, C6′), 129.3 (C12), 105.4 (C1″), 105.2 (C1′), 90.8 (C3), 82.0 (C2′), 77.8 (C5″), 77.3 (C5′), 76.5 (C3″), 76.2 (C2″), 76.1 (C3′), 73.6 (C4′), 73.5 (C4″), 63.3 (C9), 56.6 (C5), 48.4 (C18), 46.9 (C8), 45.0 (C20), 44.8 (C14), 42.7 (C19), 40.9 (C4), 40.6 (C1), 38.9 (C22), 38.3 (C10), 34.9 (C7), 33.1 (C17), 32.3 (C21), 29.5 (C29), 28.7 (C23), 27.9 (C28), 27.7 (C16), 26.9 (C2), 26.3 (C15), 24.2 (C27), 19.6 (C26), 18.7 (C6), 17.6 (C24), 17.4 (C25); 2 $CH_3SCH_2CH_2CH(NH)COOCH_3$: 174.3 (C=O), 173.6 (C=O), 53.4 (CH), 53.1 (CH), 52.8 (OCH_3), 52.6 (OCH_3), 31.7 (CH_2), 31.5 (CH_2), 31.4 (CH_2), 31.2 (CH_2), 15.7 (SCH_3), 15.6 (SCH_3). Found, %: N 2.46; S 5.64, $C_{54}H_{84}O_{18}N_2S_2$. Calc., %: N 2.52; S 5.75.

TABLE 1. Effect of Compounds on Blood Glucose Level of Rats with Alloxan Diabetes

No.	Compound	Dose, mg/kg	Blood glucose level, mM	Hypoglycemic activity, %
1	Control (diabetes), $n = 9$	-	10.1 ± 1.71	-
2	Intact, $n = 5$	-	6.8 ± 0.58	-
3	GA, $n = 8$	100	6.5 ± 1.03*	35.0
4	1Na-GA (**II**), $n = 8$	100	9.3 ± 0.95	7.9
5	Na-2Li (III), $n = 8$	100	9.5 ± 1.20	5.9
6	Na-2Li (**III**), $n = 8$	100	5.8 ± 0.62*	42.6
7	Conjugate (**IV**), $n = 8$	250	6.7 ± 0.29*	33.7

n is the number of animals in the group; * data statistically significant vs. control group with diabetes ($p < 0.05$).

EXPERIMENTAL PHARMACOLOGICAL PART

The hypoglycemic activity of GA and its derivatives **II**-**IV** was studied using 56 male Wistar rats (160 – 180 g) with intraperitoneal administration of alloxan solution (5%) at a dose of 170 mg/kg [26]. The animals were deprived of feed with access to water for 1 d before reproducing diabetes and during the experiments.

The animals were kept under standard vivarium conditions with feed and water available *ad libitum*. Animal experiments were conducted in compliance with international rules [*European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes*, No. 123, Strasbourg, 1986; *Protocol of Amendment to the European Convention for the Protection of Vertebrate Animals Use for Experimental and Other Scientific Purposes*, Strasbourg, June 22, 1998] and were approved by the

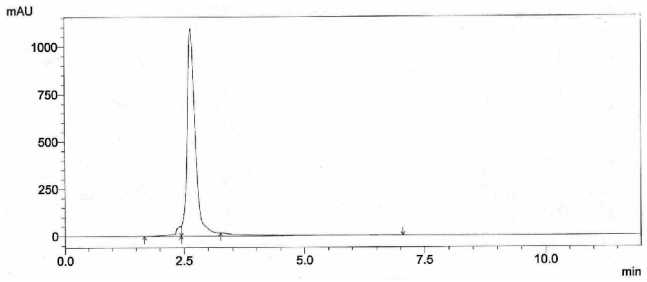


Fig. 4. HPLC chromatogram of GA conjugate with MetOMe (**IV**). Vydac C18 column (4.6 × 250 mm); mobile phase MeOH; flow rate, 1 mL/min; spectrophotometric diode-array detector at λ = 254 nm. RT = 2.65 min; content of main compound 96.2 ± 0.5%.

Biomedical Ethics Commission of Ufa Institute of Chemistry, an independent structural subdivision of UFRC, RAS.

The test compounds were administered perorally at a dose of 100 mg/kg one hour before injecting alloxan. The blood glucose concentration of the animals was determined using a glucose-oxidase biosensor method and a Onetouch Ultra handheld blood glucose meter (Johnson & Johnson, USA) 120 min after alloxan injection. Hypoglycemic activity of the compounds was assessed from the drop of blood glucose level relative to the control animals with diabetes. Experimental results were processed using the Statistica 10 program (StatSoft). Data were expressed as means and their errors ($m \pm SEM$). Intergroup comparisons were made using the Student t-criterion and were considered statistically significant for $p < 0.05$. Table 1 presents the experimental data. The reference drug was Glucobay (acarbose, tablets, 100 mg, Bayer, competitive inhibitor of intestinal á-glucosidase) at a dose of 250 mg/kg [27].

Acute toxicity of conjugate **IV** was determined using inbred female white mice (18 – 20 g) (five animals per group) and a single intragastric injection. Conjugate **IV** was injected at doses of 500, 1000, and 2000 mg/kg. The animals were observed for 14 d. Premature death of animals, the general condition, locomotor activity, and demand for feed and water were recorded. Toxicity parameters were calculated according to recommendations for studying the general toxicity of biologically active compounds [28].

RESULTS AND DISCUSSION

Table 1 presents the experimental results.

Table 1 shows that GA possessed hypoglycemic activity and reduced the blood glucose level of rats with experimental alloxan diabetes 120 min after peroral administration by 35.5% to the level of intact animals (without diabetes). GA salts **II** and **III** exhibited weak hypoglycemic activity. GA conjugate **IV** showed higher hypoglycemic activity than GA and acarbose, inhibiting the glucose rise by 42.6% relative to the control with diabetes.

The acute toxicity of **IV** was determined using inbred white mice and intragastric injection [28]. The doses were chosen based on acute toxicity data for highly purified GA (LD_{50} 5000 mg/kg) [11]. Conjugate **IV** at a dose up to 2000 mg/kg did not show toxic effects and could be assigned to class III low-toxicity compounds.

Thus, chemical modification of GA by adding *L*-methionine methyl ester to the carbohydrate part of the glycoside had a substantial effect on the ability of the glycoside to inhibit hyperglycemia in animals with an alloxan diabetes model. Conjugate **IV** was a low-toxicity compound with pronounced activity in the alloxan diabetes model in rats that was comparable to the hypoglycemic effect of acarbose. This GA derivative was promising for expanded pharmacological studies as a new antidiabetic agent.

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