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Securigenin glycosides as hypoglycemic principles of *Securigera securidaca* seeds

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Abstract Seeds of *Securigera securidaca* (Fabaceae) are used in Iranian folk medicine as an antidiabetic treatment. In this study, the antihyperglycemic activity of chloroform and methanol fractions (CF and MF) from *S. securidaca* seed extract was investigated and their bioactive constituents were identified. The antidiabetic effects of fractions were assessed by streptozocin-induced diabetic Naval Medical Research Institute mice. The hypoglycemic activity of MF at 100 mg/kg and CF at 400 mg/kg was comparable with glibenclamide (3 mg/kg). MF at 400 mg/kg and CF at 600 mg/kg showed equal hypoglycemic responses to 12.5 IU/kg insulin ($P > 0.05$). Three cardiac glycosides were isolated as active constituents responsible for the hypoglycemic activity. Securigenin-3-*O*-β-glucopyranosyl-(1 → 4)-β-xylopyranoside (**1**) was a major compound in seeds. Securigenin-3-*O*-inositol-(1 → 3)-β-glucopyranosyl-(1 → 4)-β-xylopyranoside (**2**) and securigenin-3-*O*-α-rhamnopyranosyl-(1 → 4)-α-glucopyranoside (**3**) were found as new natural products. When **1–3** were tested at 10 mg/kg there was a significant reduction of blood glucose levels in diabetic mice, comparable to that of 3 mg/kg glibenclamide ($P > 0.05$). The hypoglycemic effect was due to an increase in insulin secretion; the insulin levels in the diabetic mice significantly improved and were comparable with those in healthy animals ($P > 0.05$). Compounds responsible for the hypoglycemic properties of *S. securidaca* seeds were identified as cardiac glycosides and were found to act via an increase of insulin levels in a diabetic mouse model.

Keywords *Securigera securidaca* · Antidiabetic activity · Cardenolides · Securigenin glycosides · Insulin secretion

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Introduction

Securigera securidaca (L.) Deg. et Dorf (Fabaceae) is a native plant of Iran [1]. Seeds of *S. securidaca* are traditionally used in the southern part of Iran (Fars Province) as a remedy to control diabetes. In recent years, this traditional use became known throughout the entire country and the seeds are now widely available on local markets. Use of *S. securidaca* does not seem to be restricted to Iran, since consumption as an antidiabetic agent has also been reported from India and Egypt [2, 3].

The antihyperglycemic activity of *S. securidaca* seeds has been confirmed by several in vivo studies [3–8]. Intraperitoneal and oral administration of aqueous infusions and

Table 1 ^{1}H (500 MHz, CD$_3$OD) and ^{13}C NMR (125 MHZ, CD$_3$OD) data of compounds **1–3**

Position	Compound **1** δ H	δ C	Compound **2** δ H	δ C	Compound **3** δ H	δ C
1	1.30, *m*; 2.38, *m*	29.7	1.35, *m*; 2.44, *m*	29.6	1.32, *m*; 2.40, *m*	29.6
2	1.34, *m*; 1.99, *m*	28.7	1.39, *m*; 2.04, *m*	28.7	1.37, *m*; 2.01, *m*	28.4
3	4.16, *t*, *J* = 7.4 Hz	75.8	4.21, *t*, *J* = 7.4 Hz	75.8	3.21, *dd*, *J* = 8.9, 8.0 Hz	76.0
4	5.78, *s*	127.7	5.83, *s*	127.7	5.70, *s*	127.3
5	–	140.0	–	140.0	–	140.1
6	2.28, *m*; 2.33, *m*	34.1	2.34, *m*; 2.39, *m*	34.1	2.30, *m*; 2.35, *m*	34.1
7	1.10, *m*; 2.15, *m*	29.3	1.15, *m*; 2.21, *m*	29.3	1.13, *m*; 2.19, *m*	29.3
8	1.84, *m*	43.9	1.89, *m*	43.9	1.86, *m*	43.9
9	1.27, *m*	50.8	1.32, *m*	50.8	1.29, *m*	50.7
10	–	54.8	–	54.8	–	54.8
11	1.39, *m*; 1.65, *m*	22.7	1.43, *m*; 1.70, *m*	22.7	1.41, *m*; 1.68, *m*	22.7
12	1.36, *m*; 1.49, *m*	40.5	1.41, *m*; 1.54, *m*	40.5	1.38, *m*; 1.52, *m*	40.5
13	–	50.8	–	50.8	–	50.7
14	–	85.6	–	85.6	–	85.7
15	1.58, *m*; 1.99, *m*	32.7	1.64, *m*; 2.04, *m*	32.7	1.61, *m*; 2.01, *m*	32.7
16	1.84, *m*; 2.09, *m*	27.9	1.89, *m*; 2.15, *m*	27.9	1.86, *m*; 2.13, *m*	27.9
17	2.78, *dd*, *J* = 9.5, 5.6 Hz	51.7	2.84, *dd*, *J* = 9.5, 5.6 Hz	51.7	2.81, *dd*, *J* = 9.5, 5.6 Hz	51.6
18	0.85, *s*	16.2	0.90, *s*	16.2	0.85, *s*	16.2
19	9.78, *s*	205.4	9.83, *s*	205.5	9.83, *s*	205.5
20	–	178.2	–	178.2	–	178.2
21	4.87, *dd*, *J* = 18.4, 1.6 Hz; 4.98, *dd*, *J* = 18.4, 1.6 Hz	75.3	4.93, *dd*, *J* = 18.4, 1.6 Hz; 5.04, *dd*, *J* = 18.4, 1.6 Hz	75.3	4.91, *dd*, *J* = 18.4, 1.6 Hz; 5.02, *dd*, *J* = 18.4, 1.6 Hz	75.3
22	5.87, *s*	117.9	5.93, *s*	117.9	5.90, *s*	117.9
23	–	177.2	–	177.2	–	177.3
1′	4.34, *d*, *J* = 7.9 Hz	104.1	4.40, *d*, *J* = 7.9 Hz	104.1	4.88, *d*, *J* = 1.5 Hz	100.8
2′	3.14, *m*	74.7	3.19, *m*	74.7	3.82, *dd*, *J* = 3.1, 1.6 Hz	72.4
3′	3.28, *m*	78.0	3.33, *m*	78.0	4.14, *m*	74.0
4′	3.63, *m*	78.5	3.70, *m*	78.5	3.63, *t*, *J* = 9.4 Hz	83.4
5′	3.26, *m*; 3.96, *dd*, *J* = 11.7, 5.3 Hz	64.5	3.31, *m*; 4.02, *dd*, *J* = 11.9, 2.0 Hz	64.5	3.31, *m*	71.4
6′	–	–	–	–	3.85, *m*; 3.87, *m*	62.6
1″	4.32, *d*, *J* = 8.0 Hz	103.4	4.38, *d*, *J* = 8.0 Hz	103.4	4.59, br*s*	105.6
2″	3.16, *m*	74.6	3.21, *m*	74.6	3.31, *m*	77.9
3″	3.34, *m*	77.7	3.39, *m*	77.7	3.40, *m*	78.0
4″	3.24, *m*	71.4	3.29, *m*	71.4	3.86, *m*	72.4
5″	3.43, *t*, *J* = 9.0 Hz	76.0	3.49, *t*, *J* = 9.0 Hz	76.0	3.70, *m*	68.7
6″	3.60, *m*; 3.83, *dd*, *J* = 11.9, 2.0 Hz	62.6	3.66, *m*; 3.89, *dd*, *J* = 11.9, 2.0 Hz	62.6	–	–
6″-Me	–	–	–	–	1.31, *d*, *J* = 6.5 Hz	18.1
1‴	–	–	3.27, *m*	84.8	–	–
2‴	–	–	3.71, *m*	72.5	–	–
3‴	–	–	3.63, *m*	60.8	–	–
4‴	–	–	3.91, *m*	73.7	–	–
5‴	–	–	3.88, *m*	73.4	–	–
6‴	–	–	3.61, *m*	74.2	–	–

Compound **1**: Securigenin-3-*O*-β-glucopyranosyl-(1 → 4)-β-xylopyranoside; Compound **2**: Securigenin-3-*O*-inositol-(1 → 3)-β-glucopyranosyl-(1 → 4)-β-xylopyranoside; Compound **3**: Securigenin-3-*O*-α-rhamnopyranosyl-(1 → 4)-α-glucopyranoside

ethanol extracts significantly reduced blood glucose levels in diabetic animals while the extracts were devoid of a hypoglycemic effect in normoglycemic animals [5]. In another study, administration of a chloroform extract in normal rats reduced fasting blood sugar and improved glucose tolerance in a dose-dependent manner, accompanied with increased food consumption, body weight, and glycogen content of the liver [6]. An aqueous extract of *S. securidaca* seeds decreased erythrocyte catalase activity as an antioxidant defense mechanism in diabetic animals [9]. In addition, seeds of *S. securidaca* reportedly also possess other pharmacological activities, such as hypolipidemic, chronotropic, gastroprotective, antinociceptive, antiepileptic and cytotoxic properties [2, 10–15].

Despite the traditional consumption of *S. securidaca* seeds as an antidiabetic remedy in Iran and other countries, and pharmacological studies with crude extracts, little is known about the phytochemical composition of the seeds and other plant parts of *S. securidaca*. Flavonoids and coumarins were reported as major constituents of aerial parts, while cardenolides and dihydrobenzofuran derivatives were found in seeds [2, 15–18]. However, the compounds responsible for the antidiabetic activity remain unknown. Here, we confirm the antidiabetic activity of seed extracts and report on the isolation and structure elucidation, and pharmacological testing of antihyperglycemic constituents in *S. securidaca* seeds.

Materials and methods

Chemicals

Streptozocin (STZ) from Sigma Chemical Co. (USA), glibenclamide from Pursina (Iran), and NPH insulin from Exir Co. (Iran) were purchased. Solvents used for extraction and open column chromatography were of technical grade and were purified by distillation prior to use.

Plant material

Seeds of *S. securidaca* were collected in September 2012 from Fars Province of Iran and were dried in shade. The plant was identified by Dr. Gh. Amin and a voucher specimen was deposited at the Herbarium of Faculty of Pharmacy, Tehran University of Medical Sciences, Iran (6740-TEH).

Extraction and fractionation

The dried seeds of *S. securidaca* (1790 g) were powdered and macerated with 80% methanol at room temperature.

The extract was concentrated under reduced pressure, and remaining water was removed by freeze drying. The crude extract (323 g) was re-extracted with chloroform to afford chloroform fraction (CF; 80 g), and the residue named methanol fraction (MF; 243 g). The dried fractions were kept at 4 °C prior to testing.

Animals

Male Naval Medical Research Institute (NMRI) mice weighing between 20 and 25 g were purchased from the Pasteur Institute (Tehran, Iran). They were kept at an ambient temperature (25 ± 2 °C) in a 12-h light/dark cycle. All animals had free access to laboratory rodent diet and water. The animals were allowed to acclimatize to the laboratory environment for a week. All experimental procedures followed the principles of laboratory animal care and were carried out according to a protocol approved by the Animal Ethics Committee of the Toxicology and Pharmacology Department, Faculty of Pharmacy, Tehran University of Medical Sciences.

Induction of diabetes in mice

Diabetes mellitus type II was induced by a single intraperitoneal (i.p.) injection of 200 mg/kg STZ to overnight-fasted mice [19]. STZ was freshly dissolved in 0.05 M phosphate buffer (pH 4) prior to injection. Three days later, blood samples were obtained from the tips of mice tails, and glucose levels were determined using a glucometer (Accu Chek Sensor; Roche, Germany). Mice with fasting blood glucose of ≥ 250 mg/dl were considered as diabetic and were used for the studies.

Evaluation of antidiabetic activity

Animals were randomly divided into 13 groups, each containing 6 mice. Freshly prepared dilutions of 100–600 mg/kg of fractions, and 10 mg/kg of pure compounds were administered by i.p. injection in a fixed volume of 0.5 ml normal saline. Normal control and diabetic control groups were treated with normal saline. NPH insulin (12.5 IU/kg) and glibenclamide (3 mg/kg) were applied as positive controls [20]. Blood glucose was measured immediately before treatment (t_0), and after treatment at four time points (1, 2, 4 and 6 h). For pure compounds, heart blood was also collected after 4 h for measurement of insulin levels. Results were calculated as mean ± SD. Statistical significance was evaluated by ANOVA and Tukey posthoc tests. $P < 0.05$ was considered significant.

Isolation and purification of active constituents

A portion of CF (35 g) was separated by column chromatography on silicagel (7.0 × 16.0 cm), using a step gradient of petroleum ether/ethyl acetate (10:0, 9:1, etc.), followed by ethyl acetate/methanol (9:1, 5:5, etc.) and methanol (M). Eleven fractions (CF_1–CF_{11}) were obtained on the basis of thin-layer chromatography (TLC) patterns. CF_9 (3 g) was submitted to column chromatography on silicagel (3 × 53 cm) using a step gradient of $CHCl_3$/MeOH (9:1, 8:2, 7:3, 6:4, 2:8, 0:10). Ten subfractions were obtained (CF_{9-1} to CF_{9-10}). Compound **1** (45.7 mg) was isolated from subfraction CF_{9-7} (838 mg) by chromatography on Sephadex LH-20 (2 × 83 cm) (mobile phase $CHCl_3$/MeOH 4:6) and on an RP-18 column (1.5 × 23 cm) (mobile phase H_2O/MeOH 5:5). Subfraction CF_8 (504.4 mg) was separated on a silicagel column (2 × 39 cm) using a step gradient of $CHCl_3$/MeOH (9:1, 7:3, 5:5, 3:7, 0:10) to afford subfractions CF_{8-1} to CF_{8-4}. CF_{8-3} (114.8 mg) was further separated on a silicagel column (0.8 × 30 cm) eluted with $CHCl_3$:MeOH (8:2) to give 4 subfractions (CF_{8-3-1} to CF_{8-3-4}). Compound **2** (25.9 mg) was isolated from CF_{8-3-4} (28.4 mg) using a Sephadex LH-20 column (1.2 × 67 cm) and methanol as eluant.

A portion of MF (25 g) was submitted to column chromatography on an RP-18 (4 × 13 cm) column, using a gradient of aqueous methanol (20–80% MeOH) followed by methanol/ethyl acetate (80:20). Eight subfractions (MF_1–MF_8) were achieved on the basis of TLC patterns. MF_4 (100 mg) was further fractionated on an RP-18 column (2.5 × 17.5 cm) eluted with a gradient of aqueous methanol (40–70% MeOH). Five subfractions (MF_{4-1} to MF_{4-5}) were obtained. Compound **3** (22.5 mg) was purified from MF_{4-4} (69.6 mg) using Sephadex LH-20 (1.2 × 67 cm), and methanol as the mobile phase.

Characterization of compounds

Structures were established with the aid of 1D and 2D NMR, ESIMS and EIMS data. Absolute configuration of compounds **1** and **2** were determined by electronic circular dichroism (ECD), and X-ray crystallographic analysis of **1**.

Sugar analysis

Sugars were identified after acid hydrolysis [20]. Glycosides (10 mg) were heated on a steam bath in 10 ml HCl (2N) for 45 min. Solutions were cooled and extracted with diethyl ether. The aqueous phase was chromatographed on Whatman No. 1 paper with ethyl acetate:pyridine:water (12:5:4) as solvent. Glucose, galactose, rhamnose, mannose, xylose and myoinositol were used as reference compounds. The chromatogram was sprayed with *p*-anisidine hydrochloride/sodium hydrosulfite reagent and heated for 10 min until brown spots of sugars appeared.

X-ray crystallography

Crystal of compound **1** was measured on a Bruker Kappa Apex2 diffractometer. Apex2 software [21] was used for data collection and integration. The structure was solved by direct methods using the program SIR92 [22]. Least-squares refinement against F was carried out on all non-hydrogen atoms using the program CRYSTALS [23]. Chebychev polynomial weights [24, 25] were used to complete the refinement. Plots were produced using MERCURY [26]. Detailed experimental data are provided in Supplementary material.

Computational methods

Conformational analysis for the aglycon of **1** was performed with Schrödinger Macro Model 9.1 (Schrödinger LLC, New York, USA) employing the OPLS2005 (optimized potential for liquid simulations) force field in H_2O. Conformers within a 2 kcal/mol energy window from the global minimum were selected for geometrical optimization and energy calculation applying DFT-B3LYP/6-31 G** basis set in the gas phase with the Gaussian 09 program package. Vibrational evaluation was performed at the same level to confirm minima. Excitation energy (denoted by wavelength in nm), rotatory strength dipole velocity (R_{vel}), and dipole length (R_{len}) were calculated in MeOH by TDDFT/CAM-B3LYP/6-31 G**, using the SCRF method with the CPCM model. ECD curves were obtained on the basis of rotator strengths with a half-band of 0.25 eV using Spec Dis v1.61.

Results

Spectral analysis

The isolated compounds were identified using different spectroscopic methods (Fig. 1).

Securidaside:Securigenin-3-*O*-β-glucopyranosyl-(1 → 4)-β-xylopyranoside (**1**); white needles (recrystallized with anhydrous methanol); UV (MeOH) λ_{max}: 238 and 312 nm; ECD (MeOH, c 0.6 mM, 0.1 cm), $[\theta]_{223} = -5515$, $[\theta]_{242} = 5706$, $[\theta]_{314} = 6332$; ESIMS *m/z* 703.5 $[M + Na]^+$, 719.3 $[M + K]^+$, 725.9 $[M + HCOO]^-$; IR (KBr) v_{max} 3444, 2924, 2723, 1740, 1725, 1623, 1461, 1379, 1167, 756 cm^{-1}.

Securigenin-3-*O*-inositol-(1 → 3)-β-glucopyranosyl-(1 → 4)-β-xylopyranoside (**2**); white needles (recrystallized with anhydrous methanol); UV (MeOH) λ_{max}:
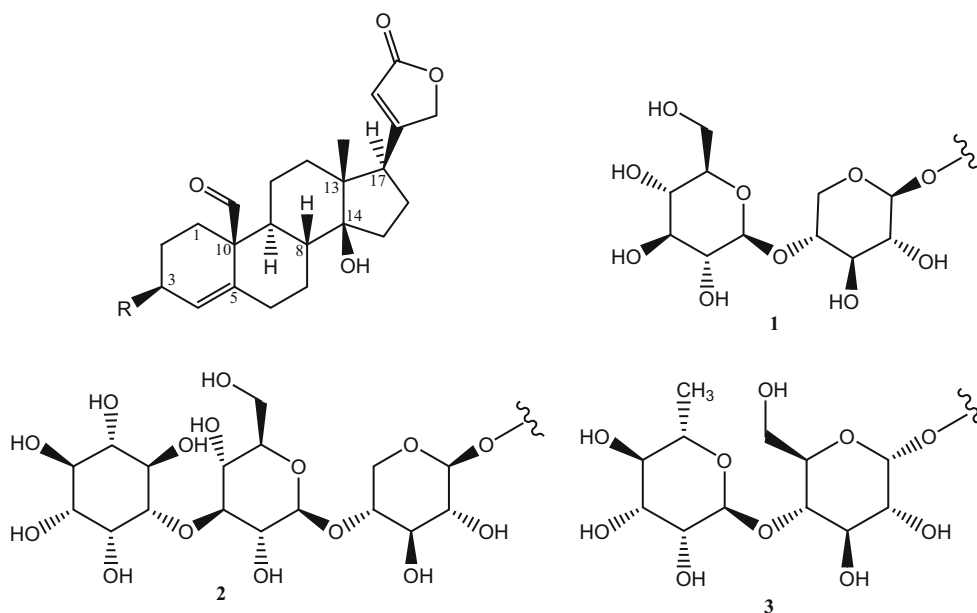
Fig. 1 Structures of cardenolides **1-3**; Securigenin-3-*O*-β-glucopyranosyl-(1 → 4)-β- xylopyranoside **1**; Securigenin-3-*O*-inositol-(1 → 3)-β-glucopyranosyl-(1 → 4)-β-xylopyranoside **2**; Securigenin-3-*O*-α-rhamnopyranosyl-(1 → 4)-α-glucopyranoside **3**

238 and 312 nm; ECD (MeOH, c 0.3 mM, 0.1 cm), $[\theta]_{223} = -2349$, $[\theta]_{242} = 13944$, $[\theta]_{314} = 5545$.

Securigenin-3-*O*-α-rhamnopyranosyl-(1 → 4)-α-glucopyranoside (**3**); white needles (recrystallized with anhydrous methanol); UV (MeOH) $\lambda_{max:}$ 238 and 312 nm.

^{1}H NMR and ^{13}C NMR data of compound **1–3** were recorded in Table 1.

Antidiabetic activities

Antidiabetic activities of seed fractions of *S. securidaca* were investigated in STZ-induced diabetic mice. Hypoglycemic activities were assessed at four time points (1, 2, 4, 6 h after i.p. injection) (Table 2). The activity of MF at 100–500 mg/kg was equivalent to that of 3 mg/kg glibenclamide ($P > 0.05$); this effect was dose-dependent. MF at 400 and 500 mg/kg gave an antihyperglycemic response that was comparable to that of 12.5 IU/kg insulin ($P = 0.23$ and 0.43, respectively). CF at 400 mg/kg showed a hypoglycemic effect comparable to glibenclamide ($P = 0.23$). There was no significant difference in blood sugar reduction percentage of 600 mg/kg of CF and glibenclamide ($P = 0.68$) or insulin ($P = 0.12$). Interestingly, onset of action (beginning of blood sugar reduction) for CF was ≤1 h, while glibenclamide showed a similar effect after 6 h.

The antidiabetic activity of pure isolated compounds was studied at 10 mg/kg BW. Securigenin glycosides **1–3** reduced blood glucose equivalent to glibenclamide ($P > 0.05$) (Table 3). Blood glucose levels in groups treated with securigenin glycosides and in the group treated with glibenclamide were reduced by approximately 40%

after 4 and 6 h, respectively. The onset of action of securigenin glycosides was faster than with glibenclamide. Blood glucose levels in animals treated with compounds **1–3** were significantly lower than for the diabetic control group. Insulin levels in diabetic mice treated with securigenin glycosides were elevated up to normal levels and were comparable with those in non-diabetic animals ($P > 0.05$). These findings were in line with previous observations with seed extracts of *S. securidaca* [3, 5].

Conformational analysis and X-ray crystallography

The experimental ECD spectrum of **1** showed two sequential positive cotton effects (CEs) at 314 and 242 nm, along with a negative CE at 223 nm. ECD spectra of cardenolides have been previously studied [27]. Hence, the spectra of **1** could not be compared with references. Therefore, we calculated the ECD spectrum of **1** and compared with experimental data. Detailed experimental data are provided in Supplementary Material. Comparison of experimental data showed an excellent fit with calculated data of the 3*S*, 8*R*, 9*S*, 10*S*, 13*R*, 14*S*, 17*R* stereoisomer (Fig. 2).

Configuration of sugars, and inter-glycosidic linkage in **1** was confirmed by single-crystal X-ray diffraction analysis of the compound (*Cu/Kα* radiation) (Fig. 3). Crystal data for compound (**1**): formula $C_{37}H_{61.46}O_{17.73}$, $M = 790.00$, $F(000) = 1701.156$, colorless needle, size 0.030–0.050–0.270 mm^3, orthorhombic, space group P 2$_1$ 2$_1$ 2$_1$, $Z = 4$, $a = 6.3288(5)$ Å, $b = 18.0687(13)$ Å, $c = 34.032(2)$ Å, $\alpha = 90°$, $\beta = 90°$, $\gamma = 90°$, $V = 3891.7(5)$ Å^3, $D_{calc.} = 1.348$ Mg m^{-3}. The data

Table 2 Hypoglycemic effects of different fractions of *S. securidaca* seeds in streptozocin-induced diabetic mice

Samples	Time (h)				
	0	1	2	4	6
MF 100 mg/kg	373.7 ± 8.8	311.3 ± 6.5	281.8 ± 5.9	257.5 ± 5.6	223.3 ± 6.0[a]
MF 200 mg/kg	348.8 ± 6.9	290.2 ± 6.1	257.8 ± 5.8	232.5 ± 6.2	218.3 ± 5.3[a]
MF 300 mg/kg	359.5 ± 4.9	299.8 ± 8.4	251.5 ± 5.1	196.5 ± 4.5	158.2 ± 3.2[a]
MF 400 mg/kg	355.7 ± 6.1	199.0 ± 3.6	167.2 ± 3.1	139.8 ± 3.6	111.7 ± 2.1[a,b]
MF 500 mg/kg	347.2 ± 8.2	273.5 ± 8.2	194.2 ± 3.6	127.0 ± 3.6	69.0 ± 1.7[a,b]
CF 300 mg/kg	398.8 ± 6.0	363.8 ± 4.4	331.7 ± 3.2	297.0 ± 1.9	255.5 ± 2.7
CF 400 mg/kg	349.2 ± 6.2	317.3 ± 3.5	277.3 ± 3.0	248.7 ± 1.7	213.2 ± 1.8[a]
CF 600 mg/kg	366.2 ± 8.4	270.3 ± 6.3	207.8 ± 4.2	161.0 ± 3.3	113.2 ± 0.9[a,b]
Glibenclamide 3 mg/kg	283.0 ± 6.8	255.5 ± 6.7	253.6 ± 8.9	242.8 ± 5.2	183.2 ± 7.4
NPH insulin 12.5 IU/kg	307.0 ± 7.2	111.8 ± 5.4	46.0 ± 3.4	39.5 ± 5.4	35.0 ± 7.2
Normal saline	309.2 ± 5.4	291.0 ± 5.1	291.0 ± 4.4	248.5 ± 6.4	208.6 ± 8.3

Results demonstrated as mean of blood glucose level (mg/dl) $\pm$ SD

MF methanol fraction, *CF* chloroform fraction

[a] There was no significant difference between remarked group and glibenclamide in a 6-h period ($P > 0.05$)

[b] There was no significant difference between remarked group and insulin in a 6-h period ($P > 0.05$)

Table 3 Antidiabetic activity of pure compounds (10 mg/kg, i.p.) and controls

Time (h):	Groups			
	Blood sugar reduction%			Insulin level (ng/ml)
	1	2	4	4
Compound **1**	14.6 ± 1.1	10.2 ± 1.4	43.9 ± 2.8[a]	0.61 ± 0.11[b]
Compound **2**	19.1 ± 3.5	18.0 ± 1.2	48.6 ± 2.5[a]	0.71 ± 0.18[b]
Compound **3**	13.5 ± 1.6	12.5 ± 1.0	33.0 ± 1.3[a]	0.59 ± 0.09[b]
Glibenclamide (3 mg/kg)	9.7 ± 2.2	10.4 ± 3.8	14.2 ± 4.5	0.48 ± 0.12
Insulin NPH (12.5 IU/kg)	63.6 ± 2.1	85.0 ± 4.6	87.1 ± 1.9	n.e
Diabetic control (NS)	13.0 ± 1.0	18.1 ± 4.3	14.7 ± 3.1	0.36 ± 0.18
Normal mice (NS)	-3.7 ± 8.0	0.7 ± 2.0	1.6 ± 3.6	0.53 ± 0.11

Results demonstrated as mean of blood glucose level (mg/dl) $\pm$ SD and as mean of blood glucose reduction% $\pm$ SD

NS normal saline, *n.e* not examined

[a] Comparable with glibenclamide with $P > 0.05$

[b] Comparable with normal mice with $P > 0.05$

Compound **1**: Securigenin-3-*O*-β-glucopyranosyl-(1 → 4)-β-xylopyranoside; Compound **2**: Securigenin-3-*O*-inositol-(1 → 3)-β-glucopyranosyl-(1 → 4)-β-xylopyranoside; Compound **3**: Securigenin-3-*O*-α-rhamnopyranosyl-(1 → 4)-α-glucopyranoside

unambiguously confirmed the structure of **1**. The crystallographic data for the aglycon structure have been deposited with the Cambridge Crystallographic Data Center (No 1034938).

Discussion

S. securidaca has a long history in folk medicine as an antidiabetic, but the nature of the active constituent(s) and molecular modes of action have not been established. We confirmed the activity of fractions obtained from seed extracts, and isolated three cardenolide glycosides as active compounds. Compounds **2** and **3** are reported here for the first time, while compound **1** (securidaside) has been previously reported from *S. securidaca*. We were able to show that these cardiac glycosides decrease blood glucose level due to an increase in blood insulin level, and thereby provide a rational explanation for the use of this traditional remedy.

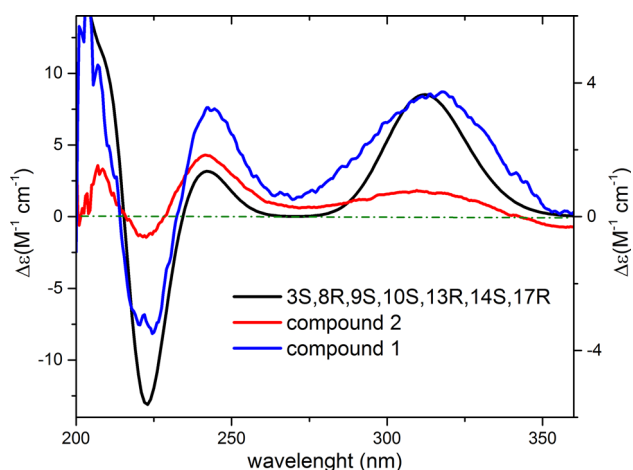The structure of compound **1** was confirmed as securidaside (securigenin-3-**O**-β-glucopyranosyl-(1 → 4)-β-

Fig. 2 Comparison of experimental and simulated ECD spectra of compounds **1** and **2** using TDDFT at CAM-B3LYP/6-31G** level, and MeOH as solvent

xylopyranoside) by 1D and 2D NMR spectra, and by comparison with previously published data [16, 28–30]. The absolute configuration of **1** was established by ECD measurements and X-ray crystallographic analysis.

The NMR data of compound **2** were very similar to those of **1**. The aglycon was also securigenin, and notable differences were detected in the signals attributable to the sugar moiety. In the ¹H-NMR spectrum, signals of anomeric protons were seen at 4.40 and 4.38 ppm (*d*, H-1′ and H-1″) and 3.27 ppm (*m*, H-1‴). The β configuration of the glucopyranosyl and xylopyranosyl units were confirmed from the doublet signal of its anomeric protons with *J* constant 7.9 and 8.0 Hz, respectively. All other proton signals appeared as multiples between 3.19 and 3.91 ppm, and the carbon signals were observed between 72.5 and 84.8 ppm. COSY and HMBC spectra revealed a securigenin-β-glucopyranosyl-(1 → 4)-

β-xylopyranosyl structure as in compound **1**, but with an additional sugar residue attached. The HMBC and COSY spectral analysis of third glycosyl moiety of compound **2** are displayed in Fig. 4. A COSY correlation between H-1‴ (3.27 ppm) and H-3″ (3.39 ppm), and a strong HMBC correlation of H-1‴ (3.27 ppm)/C-3″ (77.7 ppm) confirmed that the third sugar moiety was attached to C-3 of xylose (Fig. 4). The presence of 6 hydroxyl groups on carbons of a cyclitol ring was obvious from the ¹³C NMR chemical shifts of remaining carbons, and the residue was identified as inositol. The identity of sugars was confirmed by PC after acid hydrolysis. The ECD spectrum of **2** was identical with that of **1**, and compound **2** was thus identified as securigenin-3-*O*-inositol-(1 → 3)-β-glucopyranosyl-(1 → 4)-β-xylopyranoside, a new natural product.

NMR data of compound **3** indicated that it was also a securigenin glycoside. A notable difference was detected in the chemical shift and coupling constant of H-3 (3.21, *dd*, *J* = 8.9, 8.0 Hz) compared to compound **1** (δ H-3 4.34, 4.32, *d*, *J* = 8.0 Hz) which is due to the connection to a different conformation of sugar molecule. Signals of anomeric protons of α-glucose and α-rhamnose units appeared at 4.88 ppm (*d*, *J* = 1.5 Hz) and 4.59 ppm (*brs*), respectively. The presence of a rhamnose unit was corroborated by doublet at 1.31 ppm (*J* = 6.5 Hz, 3H) indicative of the methyl group at C-6′. The α configuration of the glucopyranosyl unit was assumed from the doublet signal of its anomeric proton at 4.88 with *J* constant 1.5 Hz in the 1H NMR spectrum while the configuration of the rhamnopyranosyl unit was deduced from the upfield shift of its C-5 (δ c 68.7) in the ¹³C NMR spectrum [31].

The structural assignment of compound **3** was confirmed by carrying out 2D NMR techniques such as HSQC, HMBC and H–H COSY. Carbons of the sugar moiety and their attached protons were correlated by an HSQC

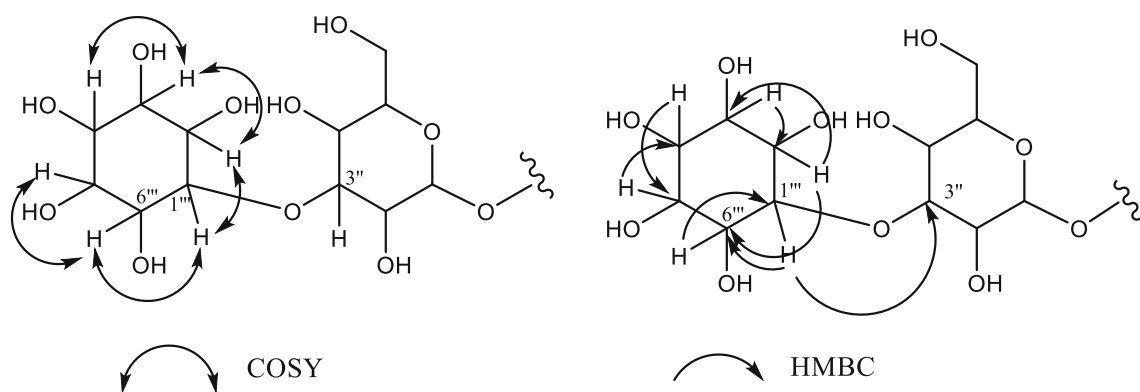**Fig. 3** X-ray structure of compound **1** (Securidaside)
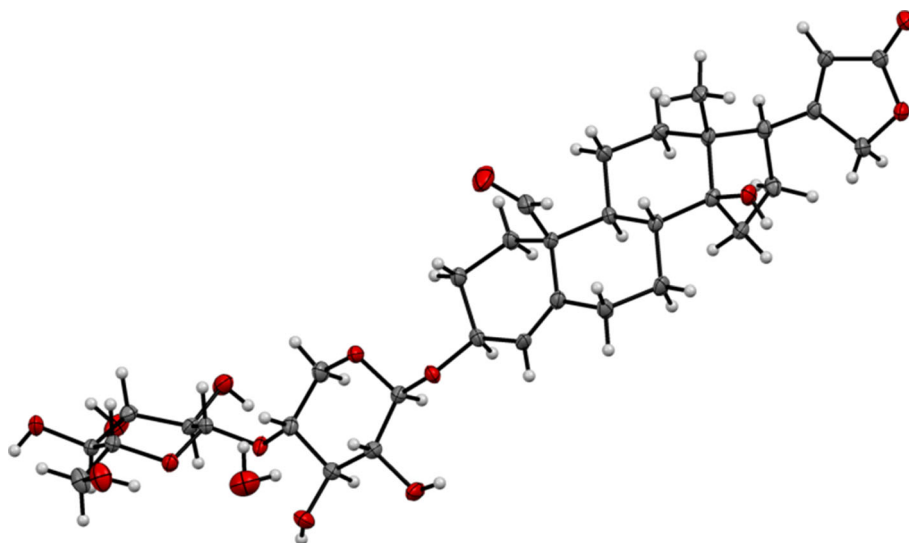
Fig. 4 H-H-COSY and HMBC correlations for the inositol-(1 → 3)-β-glucopyranosyl moiety in compound **2**

spectrum. An HMBC correlation between the anomeric proton of glucose (H-1′, 4.88 ppm) and C-3 (76.0 ppm) confirmed the 1 → 3 connectivity of the glucose unit to the aglycone. An HMBC correlation between the anomeric proton of rhamnose (H-1″, 4.59 ppm) and C-4′ (83.4 ppm) of glucose indicated a 1 → 4 connection of the two sugar units. The HMBC spectral analysis of glycosyl moiety of compound **3** displayed correlation peaks between H-1′ and C-2′, H-2′ and C-4′, H-4′ and C-2′, C-1″, C-5″, H-6′ and C-4′ of glucose sugar and H-1″ and C-4′, C-5′, H-5″ and C-3″, and methyl unit on the H-6″ of rhamnose with C-4′ and C-5″. Thus, compound **3** was identified as securigenin-3-*O*-α-rhamnopyranosyl-(1 → 4)-α-glucopyranoside, a new natural product.

Numerous plant secondary metabolites reportedly possess antidiabetic properties, including cardiac glycosides [32, 33]. Cardenolide glycoside **1**, securidaside, has been previously reported from *S. securidaca*, *Coronilla hyrcana* and *C. varia*, and showed Na+–K+ATPase inhibitory activity in a concentration range of 10^{-9}–10^{-6} mol/l. The activity was between that of ouabain and digitoxin [34]. It increased the amplitude of heart contraction, and bradycardia at 0.5–1 mg/kg, and decreased the arterial pressure and respiration at 0.2–0.25 mg/kg in mice. For securidaside, an LD_{50} of 25 mg/kg in mice has been reported after subcutaneous injection [35].

Earlier studies showed that infusion of ouabain at 1.0 µg/kg caused a significant decrease in plasma glucose and glycerol concentrations, but did not produce any marked changes in the electrocardiogram or plasma potassium [36]. Subsequently it was shown that the hypoglycemic effect of ouabain was due to an increase of insulin secretion [37]. Enhancement of insulin levels may be a reason for the apparent lack of toxicity of cardiac glycosides in diabetic animals, since insulin has been shown to interact directly with Na+–K+ATPase and thus protects from cardiac toxicity [38]. The D-glucose on the terminal position of glucose moiety of cardiac glycosides

plays an important role in the inhibition of the digitalis receptor [39]. This could be another reason which described non-toxicity of cardiac glycosides without deoxy sugars. The increase of insulin and decrease of glucose levels by cardiac glycosides were shown in our experiment, but more studies are required to prove the efficacy and lack of toxicity of this plant on the heart muscle.

Conclusions

Although the use of *S. securidaca* has a long history in folk medicine as an antidiabetic agent, the nature of its active ingredient has not been established. Our study confirmed that the seed extract and three isolated cardenolide glycosides are responsible for this activity and compounds **2** and **3** are reported here for the first time. These cardiac glycosides showed a reduction of blood sugar due to an increase of insulin level and thereby provide a rational explanation for the use of this traditional remedy.

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