Antidiabetic and Antioxidant Activities of Major Flavonoids of *Cynanchum acutum* L. (Asclepiadaceae) Growing in Egypt

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Z. Naturforsch. **63 c**, 658–662 (2008); received February 27/May 19, 2008

Seven flavonoids were isolated from the butanol fraction of the methanolic extract of the aerial parts of *Cynanchum acutum* L. (Asclepiadaceae). All of which have been isolated for the first time from the genus *Cynanchum*. Their structures were established as quercetin 3-*O*-β-galacturonopyranoside (**1**), quercetin 7-*O*-β-glucopyranoside (**2**), tamarixtin 3-*O*-β-galacturonopyranoside (**3**), kaempferol 3-*O*-β-galacturonopyranoside (**4**), 8-hydroxyquercetin 3-*O*-β-galactopyranoside (**5**), tamarixtin 3-*O*-α-rhamnopyranoside (**6**), and tamarixtin 7-*O*-α-arabinopyranoside (**7**) on the basis of their chromatographic properties, chemical and spectroscopic data. The major isolated flavonoids **1**, **2** and **3** were found to exhibit significant antioxidant and antidiabetic activities (by measuring blood glucose and insulin levels). This is the first report about the antioxidant and antidiabetic activities of compounds **1**–**3**.

Key words: Cynanchum acutum, Antioxidant, Antidiabetic

Introduction

Cynanchum acutum belongs to the family Asclepiadaceae (milk-weed family) which comprises about 2900 species in 315 genera (Boulos, 2000). The *Cynanchum* genus comprises about 200 species (Boulos, 2000) reported for their use in folk medicine as antifebrile, antitumour, antitussive, diuretic, expectorant, anticonvulsant, anodyne, and tonic agent, and is effective against chronic hepatitis (Tawfiq, 1991). *C. acutum*, native to Southern Europe, is the only *Cynanchum* species mentioned in the Egyptian flora (Täckholm, 1974). Screening of the biological activities of the total alcoholic extract of the underground organs of *C. acutum* revealed that it could inhibit the force and frequency of the intestine, heart and uterine contraction; it also exhibited a marked anti-inflammatory effect (Tawfiq, 1991). The alcoholic extract of leaves could be used as anti-inflammatory, analgesic, antipyretic, molluscicidal, insecticidal, in the treatment of cardiac arrhythmia, intestinal colic, as hypotensive and for improving respiration in asthma (El-Lithi, 1993; Abou Zeid *et al.*, 2001; Awaad, 2000). The flavonoids of *C. acutum* were tentatively identified by HPLC and chemical analysis (Abou Zeid *et al.*, 2001; Heneidak *et al.*, 2006), whereas other phytochemical studies reported the identification of some sterols (Halim *et al.*, 1990). In the present study, we report the isolation and identification of seven flavonoids from the butanol fraction (BF) of the methanolic extract of *C. acutum*. Flavonoids are known to exhibit strong antidiabetic (Singab *et al.*, 2005; Wang and Ng, 1999; Shukla *et al.*, 2004; Chylack and Cheng, 1978; Hnatyszyn *et al.*, 2002) and antioxidant activities (Rice-Evans *et al.*, 1996; Heim *et al.*, 2002; Cao *et al.*, 1997) which prompted us to test the BF and the major isolated flavonoids **1–3** for these effects.

Materials and Methods

General

UV spectra were measured using a Shimadzu UV 240 (P/N 204-58000) spectrophotometer. NMR spectra were recorded at 300 (^{1}H) and 75 MHz (^{13}C) on a Varian Mercury-300 instrument. NMR spectra were recorded in DMSO-d_6, and chemical shifts were given in δ (ppm) relative to

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TMS as internal standard. For column chromatography, Sephadex LH-20 (Pharmacia, Uppsala, Sweden), microcrystalline cellulose (Merck, Germany) and polyamide S (Fluka) were used. For paper chromatography, Whatmann No. 1 sheets (Whatmann Ltd., England) were used, while silica F_{254} (Merck) was used for TLC.

Plant material

Aerial parts of *Cynanchum acutum* were collected at Wadi El-Notron-El-Almein road, west coastal region, Egypt, during December 2005. Authentication of the plant was established by Ass. Prof. Dr. Sherif El-Khanagry, Agriculture Museum, El-Dokki, Giza, Egypt. A voucher specimen (No. C-4) is kept in the Department of Pharmacognosy, Faculty of Pharmacy, Cairo University, Egypt.

Extraction and isolation

The air-dried powdered flowering aerial parts of *C. acutum* (3 kg) were subjected to exhaustive extraction with 80% MeOH under reflux (70 °C). The total extract (200 g) was suspended in distilled water (300 ml) and successively partitioned with *n*-hexane (4 × 300 ml), chloroform (4 × 300 ml) and *n*-butanol (5 × 300 ml). The butanol fraction (BF) was evaporated to yield 30 g dry residue, which were fractionated on a polyamide column (12 × 115 cm, 400 g), eluted with H_2O followed by a gradient of H_2O/MeOH mixtures up to pure MeOH. On the basis of TLC and PC with the use of UV light, 5% $AlCl_3$, 1% $FeCl_3$, or 10% H_2SO_4 spray reagents for detection, similar fractions were pooled together to yield 8 collective fractions (I–VIII). The flavonoid-rich fractions were fractions IV and V. Fraction IV (50% MeOH/H_2O, 5 g) was chromatographed on a Sephadex LH-20 column using *n*-BuOH/isopropanol/H_2O (BIW, 4:1:5, v/v/v upper layer) for elution to afford four subfractions (i–iv). Subfraction ii showed only one retention time; it was then purified on a Sephadex LH-20 column with MeOH (eluent) to give compound **1** (2 g). Subfraction iv was chromatographed on a cellulose column with 80% MeOH/H_2O and rechromatographed on Sephadex with EtOH to yield compound **2** (300 mg).

Fraction V (60% MeOH/H_2O, 6.3 g) was chromatographed on cellulose using BIW for elution to afford two subfractions (i, ii). Subfraction i was chromatographed on a Sephadex LH-20 column with BIW to give compounds **3** (1 g) and **4** (0.5 g). Subfraction ii was chromatographed on a Sephadex LH-20 column with BIW and rechromatographed on Sephadex (70% MeOH/H_2O) to yield compounds **5** (0.6 g), **6** (40 mg), and **7** (45 mg). All separation processes were followed up by Co-TLC with $CHCl_3$/MeOH (8:2) while S_1 (*n*-BuOH/HOAc/H_2O, 4:1:5, top layer) and S_2 (15% aq. AcOH) were used for comparative and 2D-PC.

Chemicals

Alloxan (Sigma Co., USA), metformin (Cidophage®, CID Co., Giza, Egypt) and vitamin E (Pharco Pharmaceutical Co., Alexandria, Egypt).

Animals

Male Swiss albino mice (20–25 g) and adult male albino rats (130–150 g) were obtained from the animal-breeding unit of National Research Centre, El-Dokki, Giza, Egypt. All animals were kept in an air-conditioned room at (22 ± 3) °C, (55 ± 5)% humidity, 12 h light and were fed on standard laboratory diet and water *ad libitum*. In a preliminary test for the acute antidiabetic effect, 30 adult male albino rats were used after induction of diabetes. They were divided into 3 groups (each of 10). One group was kept as diabetic non-treated, the second group was treated with 100 mg kg^{-1} body weight (wt) BF, while the third group received metformin (150 mg kg^{-1} body wt).

For testing the chronic antidiabetic effect, 50 adult male albino rats were used in the experiment and divided into 5 groups (each of 10). All groups were injected intraperitoneally with a single dose of alloxan (150 mg kg^{-1} body wt) to induce hyperglycemia (Eliasson and Samet, 1969; Szkudelski, 2001). One group was kept as diabetic non-treated, the second was given metformin orally in a dose of 150 mg kg^{-1} body wt daily along the time of experiment (8 weeks), the third to fifth groups received 50 mg/kg body wt orally of compounds **1**–**3**, respectively.

Determination of LD_{50}

LD_{50} was determined according to the procedures developed by Karber (1931).

Hypoglycemic effect

The serum glucose (Trainder, 1969) and insulin levels were measured (Marchner, 1974) using Biomurex Kits (Durham, NC, USA) in alloxan-induced diabetic rats (150 mg kg^{-1} body wt), after oral administration of the BF (100 mg kg^{-1} body wt) and isolated compounds **1–3** (50 mg kg^{-1} body wt) for 8 weeks.

Measurement of GSH content

The GSH content was measured (Beutler *et al.*, 1963) in alloxan-induced diabetic rats (150 mg kg^{-1} body wt) using glutathion kits (Wak Company, Frankfurt, Germany). Vitamin E was used as reference drug.

Statistical analysis

All data were expressed as mean ± SE and the statistical significance was evaluated by one-way analysis of variance ANOVA (Sendecor and Cochrean, 1971). The values were considered to be significantly different when *P* values were less than 0.01.

Results

General

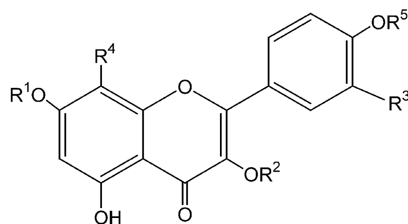
Three quercetin glycosides (**1**, **2**, **5**), three tamarixtin glycosides (**3**, **6**, **7**) and one kaempferol galacturonoside (**4**) were isolated from the BF of aerial parts of *C. acutum* after fractionation on a polyamide column, followed by several cellulose and Sephadex LH-20 columns (Fig. 1). All isolated compounds are reported for the first time for the genus *Cynanchum*. Their structures were confirmed by comparison of their chromatographic properties, chemical and spectroscopic data (UV, ^{1}H and ^{13}C NMR) with those reported in the literature (Mabry *et al.*, 1970; Agrawal and Bansal, 1989; Markham and Mohanchari, 1982).

Biological study

The investigated BF was found to be non-toxic up to a maximum soluble dose (LD_{50} = 5.6 g kg^{-1} body wt).

This study was undertaken to assess antiperoxidation properties of the BF of *C. acutum* in alloxan-induced diabetic rats through investigation of its antioxidant and antidiabetic effects. The basal levels of blood glucose of rats were not significantly different prior to alloxan administration. However, 24 h after alloxan administration, the blood glucose levels were significantly higher in rats selected for the study. In contrast, non-diabetic controls remained persistently euglycemic throughout the course of the study.

The BF was initially tested for its acute antidiabetic activity ($P < 0.01$) at a dose of 100 mg kg^{-1} body wt. It showed significant antidiabetic activity that encouraged further investigation of its major isolates for their chronic antidiabetic activity through measuring glucose and insulin levels. For the chronic activity assay (Table I), major isolates **1–3** (50 mg kg^{-1} body wt) were administered daily for 8 weeks to alloxan-induced diabetic rats. All tested compounds showed a significant antidiabetic effect that was evident from the fourth week onwards; the decrease in the blood glucose level and the increase in the insulin level were maxi-



Compound	R¹	R²	R³	R⁴	R⁵
1	H	Galacturonide	OH	H	H
2	Glucoside	H	OH	H	H
3	H	Galacturonide	OH	H	CH_3
4	H	Galacturonide	H	H	H
5	H	Galactoside	OH	OH	H
6	H	Rhamnoside	OH	H	CH_3
7	Arabinopyranoside	H	OH	H	CH_3

Fig. 1. Chemical structures of the compounds isolated from *C. acutum*.

Table I. Effect of isolates **1–3** of *C. acutum* and metformin on diabetic male albino rats (n = 10) (± SE).

Group	Zero		4 weeks		8 weeks	
	Glucose [mg/l]	Insulin [μU/ml]	Glucose [mg/l]	Insulin [μU/ml]	Glucose [mg/l]	Insulin [μU/ml]
Non-treated	257.2 ± 8.4	8.9 ± 0.3	261.3 ± 12.7^{b}	9.1 ± 0.4^{b}	265.8 ± 11.7^{b}	8.3 ± 0.6^{b}
Treated with **1**	249.4 ± 11.3	9.5 ± 0.7	196.3 ± 7.6^{ab} (21%)	20.1 ± 0.5^{ab}	123.2 ± 6.4^{ab} (50%)	26.1 ± 0.4^{ab}
Treated with **2**	268.1 ± 9.3	9.7 ± 0.2	169.4 ± 7.2^{ab} (37%)	15.3 ± 0.4^{ab}	131.2 ± 4.8^{ab} (51%)	27.2 ± 0.8^{ab}
Treated with **3**	243.1 ± 10.6	8.6 ± 0.3	172.8 ± 7.9^{ab} (28%)	17.1 ± 0.8^{ab}	138.2 ± 5.7^{ab} (43%)	29.8 ± 0.7^{ab}
Treated with metformin	255.6 ± 11.3	7.6 ± 0.3	103.6 ± 4.2^{a} (59%)	26.4 ± 0.8^{a}	82.9 ± 2.7^{a} (67%)	39.8 ± 1.1^{a}

[a] Significantly different from non-treated value at $P < 0.01$.
[b] Significantly different from metformin value at $P < 0.01$.
Values in parentheses indicate the percentage lowering of blood glucose in comparison to basal reading at zero time.

Table II. Effect of isolates **1–3** of *C. acutum* and vitamin E on diabetic male albino rats (n = 10).

Group	Blood glutathione ± SE [mg/dl]	Change from diabetic (%)
Control	36.3 ± 1.5^{b}	
Diabetic	22.2 ± 0.8^{ac}	–
Diab. treated with **1** (50 mg kg^{-1})	34.8 ± 1.1^{b}	56.7
Diab. treated with **2** (50 mg kg^{-1})	34.7 ± 0.9^{b}	56.3
Diab. treated with **3** (50 mg kg^{-1})	34.2 ± 1.3^{b}	54
Vitamin E	35.8 ± 1.4 ^{b}	61.2

[a] Significantly different from control value at $P < 0.01$.
[b] Significantly different from diabetic value at $P < 0.01$.
[c] Significantly different from vitamin E value at $P < 0.01$.

mum on completion of the eighth week. Maximum reduction in the glucose level was found in animals receiving compound **2** (51%), followed by compound **1** (50%), whereas maximum increase in the insulin level was found in animals receiving compound **3** followed by compound **2**.

To assess the antioxidant activity of the isolates **1**–**3**, the GSH level was measured in diabetic rats. GSH is the first line of defense against a prooxidant status (Pari and Latha, 2004). GSH systems may have the ability to manage oxidative stress with adaptational changes in the enzyme regulating GSH metabolism. In the present study (Table II), treatment with the major isolates of *C. acutum* significantly increased the GSH level which may in turn activate the GSH-dependant enzymes such as glutathione peroxidase and glutathione-*S*-transferase. The increase in the GSH level in all animals was not significantly different from that of vitamin E; the most effective one was compound **1** (increase by 56.7%).

Discussion

The major isolates **1**–**3** showed potent antidiabetic activity in comparison with metformin through an increase of insulin and a decrease of glucose levels. The activity of the BF in the acute antidiabetic study is likely due to its enrichment with these isolates. By the same way, all tested isolates were very potent as antioxidants through increasing GSH levels (Chin *et al.*, 2004) and were not significantly different from vitamin E. The antioxidant activity of flavonoids is based upon the structure-activity relationship, *i.e.* the π-bond conjugation over the A- and B-rings through a 4-keto group, 2,3-double bond and the aromatic-OH groups. This conjugation is responsible for the sta-

bilization of the aryloxy radical after hydrogen donation in the free radical scavenging process (Peng *et al.*, 2003). This result suggests that the antidiabetic action of the BF is mediated by an increase in the insulin level and antioxidant action on pancreatic β-cells.

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