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

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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), tamarixin 3-O-β-galacturonopyranoside (3), kaempferol 3-O-β-galacturonopyranoside (4), 8-hydroxyquercetin 3-O-β-galactopyranoside (5), tamarixin 3-O-α-rhamnopyranoside (6), and tamarixin 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 (1H) and 75 MHz (13C) on a Varian Mercury-300 instrument. NMR spectra were recorded in DMSO-d6, and chemical shifts were given in δ (ppm) relative to
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$_2$O followed by a gradient of H$_2$O/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$_2$SO$_4$ 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$_2$O, 5 g) was chromatographed on a Sephadex LH-20 column using $n$-BuOH/isopropanol/H$_2$O (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$_2$O and rechromatographed on Sephadex with EtOH to yield compound 2 (300 mg).

Fraction V (60% MeOH/H$_2$O, 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$_2$O) 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$_2$O, 4:1:5, top layer) and S$_2$ (15% aq. AcOH) were used for comparative and 2D-PC.

**Chemicals**

Alloxan (Sigma Co., USA), metformin (Cidoephage®, 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 Cochran, 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-
Table I. Effect of isolates 1–3 of C. acutum and metformin on diabetic male albino rats (n = 10) (± SE).

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<td></td>
<td>Zero</td>
<td>4 weeks</td>
<td>8 weeks</td>
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<tr>
<td>Non-treated</td>
<td>257.2 ± 8.4</td>
<td>8.9 ± 0.3</td>
<td>261.3 ± 12.7b</td>
<td>9.1 ± 0.4b</td>
<td>265.8 ± 11.7b</td>
<td>8.3 ± 0.6b</td>
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<tr>
<td>Treated with 1</td>
<td>249.4 ± 11.3</td>
<td>9.5 ± 0.7</td>
<td>196.3 ± 7.6ab</td>
<td>20.1 ± 0.5ab</td>
<td>123.2 ± 6.4ab</td>
<td>26.1 ± 0.4ab</td>
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<tr>
<td>Treated with 2</td>
<td>268.1 ± 9.3</td>
<td>9.7 ± 0.2</td>
<td>169.4 ± 7.2ab</td>
<td>15.3 ± 0.4ab</td>
<td>131.2 ± 4.8ab</td>
<td>27.2 ± 0.8ab</td>
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<tr>
<td>Treated with 3</td>
<td>243.1 ± 10.6</td>
<td>8.6 ± 0.3</td>
<td>172.8 ± 7.9ab</td>
<td>17.1 ± 0.8ab</td>
<td>138.2 ± 5.7ab</td>
<td>29.8 ± 0.7ab</td>
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<tr>
<td>Treated with metformin</td>
<td>255.6 ± 11.3</td>
<td>7.6 ± 0.3</td>
<td>103.6 ± 4.2a</td>
<td>26.4 ± 0.8a</td>
<td>82.9 ± 2.7a</td>
<td>39.8 ± 1.1a</td>
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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.

maximum 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.


