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ScienceDirect
Phytochemistry 68 (2007) 2087–2095
PHYTOCHEMISTRY
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Antidiabetic properties of selected Mexican copalchis of the Rubiaceae family

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Received 29 November 2006; received in revised form 16 April 2007
Available online 18 June 2007

Abstract

The extracts prepared from the stem barks of several Mexican copalchis species, including *Hintonia latiflora*, *Exostema caribaeum* and a commercial mixture of *Hintonia standleyana* and *E. caribaeum* (CM) showed significant hypoglycemic and antihyperglycemic effects. The extracts were tested in three different in vivo models using normal and streptozotocin (STZ)-induced diabetic rats. From the active extract of *H. latiflora*, 25-*O*-acetyl-3-*O*-β-D-glucopyranosyl-23,24-dihydrocucurbitacin F (**1**), an analog of 23,24-dihydrocucurbitacin F, and several known compounds (**2**–**8**) were isolated; cucurbitacin **1** was also isolated from *H. standleyana*. Oral administration of *H. latiflora* extract [100 mg/kg of body weight (bw)] and 5-*O*-β-D-glucopyranosyl-7,3′,4′-trihydroxy-4-phenylcoumarin (**5**) (30 mg/kg of bw) to STZ-induced diabetic rats, for a 30 day duration, restored blood glucose levels to normal values. The groups treated either with the active principle **5**, or the extract of *H. latiflora*, showed less body weight loss than glibenclamide-treated and diabetic control groups ($p < 0.05$). It was also demonstrated that the extract of *H. latiflora* regulated hepatic glycogen and plasma insulin levels ($p < 0.05$). These data suggest that its antihyperglycemic effect is due in part to stimulation of insulin secretion and regulation of hepatic glycogen metabolism.

Keywords: *Hintonia latiflora*; *Hintonia standleyana*; *Exostema caribaeum*; Copalchi; Rubiaceae; 4-Phenylcoumarins; Cucurbitacins; STZ-induced diabetic rats; Diabetes mellitus

1. Introduction

In Mexico, circa 10% of the population suffers non-insulin-dependent diabetes mellitus (NIDDM). As in other regions of the world, most of the Mexican people employ a variety of plant preparations for treatment of this illness (Subash-Babu and Mainzen-Prince, 2004). Among the plants highly valued for treatment of NIDDM are several of the Rubiaceae with extremely bitter stem barks. These species are commonly regarded as "copalchis" and the most important are *Hintonia latiflora* (Sesse et Mociño ex DC) Bullock (synonym: *Coutarea latiflora* Sesse et Mociño ex DC), *H. standleyana* Bullock, *Exostema caribeaum* (Jacq.) Roem et Schult and *E. mexicanum* Gray. Previous chemical investigation of these species resulted in isolation and identification of several cucurbitacins and 4-phenylcoumarins (Reher and Kraus, 1984; Mata et al., 1987, 1988, 1990a,b, 1992; Reguero et al., 1987; Guerrero-Analco et al., 2005; Argotte-Ramos et al., 2006), a phenylstyrene (Mata et al., 1992), as well as an indole monoterpenoid alkaloid (Déciga-Campos et al., 2006). From a pharmacological point of view, the antimicrobial (Rojas et al., 1992) and antiplasmodial properties of the extracts and some 4-phenylcoumarins from *H. latiflora*, *E. caribaeum* and *E. mexicanum* were also demonstrated (Noster and Kraus, 1990; Köhler et al., 2001). Additionally, the extract of *H. standleyana*, as well as its cucurbitans, induced antinociceptive effects in mice. We also demonstrated that the

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[1] Taken in part from the PhD thesis of J.A. Guerrero-Analco.

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doi:10.1016/j.phytochem.2007.05.006

extracts of both *Hintonia* species are non-toxic to mice when tested according to the Lorke procedure (Déciga-Campos et al., 2006; Déciga-Campos et al., 2007); however, the extract of *E.caribaeum* result in various toxic effects such as inducing tremor, respiratory distress as well as a decrease in motor activity (Déciga-Campos et al., 2007).

The antidiabetic properties of *H. latiflora* have been extensively investigated, with the earliest work carried out at the beginning of the 20th century at the Instituto Médico Nacional in Mexico City (Martínez, 1989). Later, researchers in Germany (Khur, 1953; Kaiser and Geyer, 1955; Slijepcevic and Kraus, 1997; Korec et al., 2000), France (Bastien, 1961), and Italy (Pinto et al., 1997) corroborated the work of the Mexican scientists using short term animal experiments. Recently, Korec and co-workers (2000), using short term experiments, reported that oral administration of an extract prepared from a commercial mixture of the stem barks of *H. latiflora* and *E. caribaeum* (CM) of uncertain origin decreased glucose levels in streptozotocin (STZ)-diabetic rats. In that study, however the natural active principles contributing to this activity were not determined. More recently, our research group studied the antidiabetic potential of *H. standleyana* (Guerrero-Analco et al., 2005); these results demonstrated the antihyperglycemic effect of an organic extract of the stem bark of *H. standleyana* and compounds **2** and **6** (Guerrero-Analco et al., 2005). The relevant issues raised in this study were: (i) the establishment of active principles of a Mexican copalchi, which turned out to be 4-phenylcoumarin glycosides and cucurbitacins, and (ii) the demonstration in a long term assay that compounds **2** and **6** reduced blood glucose levels near to normal values in STZ-induced diabetic rats.

Continuing our work on Mexican copalchis of the Rubiaceae family (Guerrero-Analco et al., 2005), we describe herein the long term antihyperglycemic effect of the extracts prepared from *H. latiflora*, *E. caribaeum* and a commercial mixture of *E. caribaeum* and *H. standleyana* (CM) that were purchased in local markets. Finally, the effect of copalchis extracts and their antihyperglycemic compounds on plasma insulin and hepatic glycogen levels were assessed on STZ-induced diabetic rats. These two experiments were performed in order to initiate the studies regarding the antidiabetic mode of action of Mexican copalchis herbal preparations and their active principles.

2. Results and discussion

As shown in Tables 1 and 2, the organic extracts (100 mg/kg and 300 mg/kg of bw) of the stem-barks of *H. latiflora*, *E. caribaeum* and CM caused significant decreases in blood glucose levels, in both normal and STZ-diabetic rats when compared with vehicle-treated groups ($p < 0.05$). The results of *H. latiflora* are thus in agreement with those previously described by Bastien (1961) and Pinto et al. (1997). In normoglycemic animals, the hypoglycemic effect of *H. latiflora* was around 24%

Table 1
Acute effect of the extracts of *H. latiflora*, *E. caribaeum* and CM (commercial mixture of copalchi) on blood glucose levels in normal rats

Test samples	Blood glucose concentration (mg/dl)						
	Dose (*per os*) mg/kg of bw	0 h	1.5 h	3 h	5 h	7 h	9 h
Control (vehicle)	–	92.5 ± 2.85	99.1 ± 3.2 (7.10)	92.4 ± 3.1 (−0.05)	88.3 ± 4.0 (−4.50)	88.9 ± 4.1 (−3.80)	88.6 ± 3.3 (−4.2)
Glibenclamide	10	89.6 ± 6.00	74.3 ± 4.2* (−17.00)	72.4 ± 4.5* (−19.20)	62.6 ± 3.4* (−30.08)	60.4 ± 3.5* (−32.61)	68.6 ± 2.4* (−23.42)
Extract of *H. latiflora*	300	88.6 ± 1.20	99.7 ± 1.7 (12.56)	82.3 ± 2.4 (−7.13)	69.6 ± 2.5* (−21.48)	68.4 ± 1.7* (−22.84)	63.0 ± 3.2* (−28.89)
Extract of *H. latiflora*	100	93.8 ± 1.34	101.5 ± 3.2 (8.24)	73.7 ± 2.3* (−21.40)	71.0 ± 2.7* (−24.29)	72.1 ± 2.3* (−23.15)	69.0 ± 4.3* (−26.45)
Extract of *H. latiflora*	30	93.3 ± 1.45	103.0 ± 4.1 (10.36)	83.2 ± 2.7 (−10.78)	73.0 ± 2.9* (−21.71)	74.8 ± 2.7* (−19.83)	73.2 ± 2.5* (−21.57)
Extract of *H. latiflora*	10	98.3 ± 3.50	106.7 ± 3.5 (8.51)	87.5 ± 4.9 (−10.98)	78.8 ± 3.9* (−19.80)	81.1 ± 3.3* (−17.60)	82.6 ± 3.2* (−16.01)
Extract of *E. caribaeum*	300	94.6 ± 4.51	96.4 ± 3.7 (2.00)	90.6 ± 2.9 (−4.12)	79.4 ± 3.0* (−16.05)	77.3 ± 2.5* (−18.25)	75.1 ± 4.6* (−20.53)
Extract of *E. caribaeum*	100	94.3 ± 2.52	95.6 ± 4.1 (1.40)	90.0 ± 3.6 (−4.50)	84.2 ± 4.3 (−10.64)	81.2 ± 4.0* (−13.83)	77.4 ± 4.2* (−17.91)
Extract of *E. caribaeum*	30	90.0 ± 2.96	97.5 ± 6.3 (8.36)	89.1 ± 4.9 (−0.95)	83.7 ± 3.8 (−6.90)	78.4 ± 3.7* (−12.80)	77.8 ± 6.3* (−13.46)
Extract of *E. caribaeum*	10	94.0 ± 2.84	103.9 ± 5.8 (10.60)	97.4 ± 5.1 (3.66)	90.7 ± 5.5 (−3.44)	87.2 ± 4.6 (−7.13)	85.7 ± 6.2 (−8.82)
Extract of CM	300	97.9 ± 8.7	87.8 ± 2.9* (−10.22)	76.9 ± 3.2* (−21.44)	74.42 ± 3.1* (−23.99)	77.90 ± 4.8* (−20.44)	79.48 ± 3.9* (−18.83)
Extract of CM	100	97.9 ± 6.6	106.9 ± 5.5 (9.16)	96.1 ± 5.1 (−1.91)	84.78 ± 4.7* (−13.47)	87.11 ± 3.2* (−11.09)	85.23 ± 5.2* (−13.01)
Extract of CM	30	96.7 ± 4.4	89.3 ± 2.8 (−7.61)	89.4 ± 3.9 (−7.49)	86.39 ± 2.7* (−10.68)	87.75 ± 4.2 (−9.28)	92.35 ± 3.9 (−4.52)
Extract of CM	10	98.7 ± 6.2	110.1 ± 4.2 (11.57)	100.1 ± 4.0 (1.40)	94.14 ± 5.2 (−4.63)	94.89 ± 6.0 (−3.87)	88.58 ± 5.3* (−10.27)

Each value is the mean ± SEM for six rats in each group.
* $p < 0.05$ significantly different ANOVA followed by Dunnett's *t*-test for comparison with respect to initial levels in each group. % Variation of glycemia are in parentheses.

Table 2
Acute effect of the extracts of *H. latiflora*, *E. caribaeum* and CM (commercial mixture of copalchi) on blood glucose levels in STZ-diabetics rats

Test samples	Blood glucose concentration (mg/dl)						
	Dose (*per os*) mg/kg of bw	0 h	1.5 h	3 h	5 h	7 h	9 h
Control (vehicle)	–	248.0 ± 9.1	290.4 ± 4.3 (17.11)	284.7 ± 3.6 (14.81)	258.9 ± 7.4 (4.37)	229.3 ± 6.7 (−7.55)	226.6 ± 5.2 (−8.64)
Glibenclamide	10	269.7 ± 9.0	271.2 ± 2.6 (0.52)	198.7 ± 6.1* (−26.34)	173.5 ± 5.5* (−35.68)	195.1 ± 3.9* (−27.68)	208.9 ± 2.4* (−22.57)
Extract of *H. latiflora*	300	271.2 ± 9.8	299.2 ± 5.6 (5.90)	269.0 ± 4.7* (−4.76)	241.7 ± 4.3* (−14.45)	232.5 ± 3.9* (−17.70)	188.1 ± 1.8* (−33.42)
Extract of *H. latiflora*	100	262 ± 3.5	290.3 ± 5.9 (11.98)	262.7 ± 2.1 (1.33)	235.6 ± 6.0 (−9.10)	212.1 ± 3.5* (−18.19)	204.8 ± 1.8* (−21.00)
Extract of *H. latiflora*	30	259.2 ± 12.4	295.3 ± 7.7 (12.69)	273.3 ± 3.0 (4.29)	265.4 ± 5.9 (1.28)	233.6 ± 4.5 (−10.82)	218.2 ± 4.3* (−16.72)
Extract of *H. latiflora*	10	282.5 ± 6.5	303.9 ± 7.4 (12.03)	266.1 ± 11.5 (−1.90)	241.1 ± 6.6 (−11.12)	239.8 ± 7.5 (−11.58)	229.7 ± 7.9* (−15.32)
Extract of *E. caribaeum*	300	275.7 ± 2.2	304.5 ± 6.6 (11.73)	248.5 ± 6.0 (−8.81)	225.0 ± 7.3* (−17.44)	222.7 ± 3.9* (−18.28)	231.0 ± 2.4* (−15.23)
Extract of *E. caribaeum*	100	277.7 ± 7.6	289.0 ± 6.0 (9.38)	243.2 ± 3.7 (−7.95)	233.2 ± 3.5* (−11.76)	219.8 ± 4.8* (−16.82)	238.3 ± 1.7 (−9.81)
Extract of *E. caribaeum*	30	264.2 ± 5.5	298.5 ± 8.3 (7.47)	257.1 ± 6.7 (−7.42)	246.1 ± 6.9* (−11.41)	259.0 ± 11.2 (−6.74)	265.3 ± 5.8 (−4.48)
Extract of *E. caribaeum*	10	272.5 ± 3.2	308.6 ± 6.3 (11.93)	251.8 ± 6.0 (−8.69)	251.3 ± 4.9 (−8.87)	265.4 ± 4.4 (−3.75)	254.7 ± 1.5 (−7.65)
Extract of CM	300	293.7 ± 2.05	341.9 ± 5.3 (14.04)	243.0 ± 5.2* (−14.73)	200.9 ± 3.8* (−27.0)	227.0 ± 2.2* (−19.38)	233.8 ± 4.2* (−17.42)
Extract of CM	100	287.5 ± 7.29	268.8 ± 5.4 (−5.54)	261.9 ± 5.3 (−7.60)	253.0 ± 6.0 (−10.22)	246.0 ± 5.9* (−12.30)	228.9 ± 5.2* (−17.38)
Extract of CM	30	284.5 ± 16.6	331.3 ± 5.2 (14.0)	285.64 ± 5.2 (0.34)	275.64 ± 5.2 (−2.34)	241.8 ± 5.3* (−12.74)	286.64 ± 5.2 (0.64)
Extract of CM	10	279.8 ± 15.0	303.4 ± 4.2 (7.15)	279.6 ± 4.1 (−0.05)	265.3 ± 4.2 (−4.40)	262.3 ± 4.3 (−5.30)	262.0 ± 4.3 (−5.38)

Each value is the mean ± SEM for six rats in each group.
* $p < 0.05$ significantly different ANOVA followed by Dunnett's *t*-test for comparison with respect to initial levels in each group. % Variation of glycemia are in parentheses.

and persisted throughout the experiment (Table 1). In diabetic animals, the highest antihyperglycemic effect of the *H. latiflora* extract was observed at doses of 100 and 300 mg/kg at 9 h (−21.0 and −33.4%, respectively) (Table 2). In the case of the extract of *E. caribaeum*, the hypoglycemic effect was around 17% in both models (Tables 1 and 2) at doses of 100 and 300 mg/kg at 7 h (−18.3% and −16.8%, respectively). Glibenclamide (10 mg/kg), used as positive control, showed maximum hypoglycemic effect at 7 h in normoglycemic animals (Table 1) and at 5 h in STZ-induced diabetic rats (Table 2).

In order to isolate the major active principles, the extract of *H. latiflora* was subjected to an extensive chromatographic fractionation. This process led to the isolation of compounds **1**–**8** identified as 25-*O*-acetyl-3-*O*-β-D-glucopyranosyl-23,24-dihydrocucurbitacin F (**1**), a previously not described cucurbitacin glycoside, 3-*O*-β-D-glucopyranosyl-23,24-dihydrocucurbitacin F (**2**), 5-*O*-β-D-glucopyranosyl-7-methoxy-3′,4′-dihydroxy-4-phenylcoumarin (**3**) 5-*O*-β-D-galactopyranosyl-7-methoxy-3′,4′-dihydroxy-4-phenylcoumarin (**4**), 5-*O*-β-D-glucopyranosyl-7,3′,4′-trihydroxy-4-phenylcoumarin (**5**), 5-*O*-[β-D-apiofuranosyl-(1 → 6)-β-D-glucopyranosyl]-7-methoxy-3′,4′-dihydroxy-4-phenyl-coumarin (**6**), 5-*O*-[β-D-xylopyranosyl-(1 → 6)-β-D-glucopyranosyl]-7-methoxy-3′,4′-dihydroxy-4-phenylcoumarin (**7**), and the alkaloid desoxycordifolinic acid (**8**). Compound **1** was also isolated from the related species *H. standleyana*. The structure of compound **1** was elucidated using spectrometric and spectroscopic methods including uni- and bi-dimensional NMR spectroscopic techniques. Compounds **2**–**8** were characterized by comparison with authentic samples (Aquino et al., 1987; Mata et al., 1987, 1988, 1990a, 1992; Reguero et al., 1987; Guerrero-Analco et al., 2005; Déciga-Campos et al., 2006).

The HRFAB mass spectrum (positive mode) of **1** showed a *quasi*-molecular ion at *m*/*z* 725.8731 $[M+H]^+$ consistent with the molecular formulae $C_{38}H_{60}O_{13}$. The NMR spectra ($CDOD_3$) were very similar to 23,24-dihydrocucurbitacin F glucoside previously isolated from *H. latiflora* and *H. standleyana* (Mata et al., 1990a; Guerrero-Analco et al., 2005). Furthermore, acid hydrolysis of compound **1** yielded 25-*O*-acetyl-23,24-dihydrocucurbitacin F (**1a**) (Reguero et al., 1987) and glucose. The ^{13}C NMR chemical shifts observed in the region of the spectrum between δ_C 107 and 60 confirmed the presence of glucose [δ_C 106.8 (C-1′), 76.2 (C-2′), 78.5 (C-3′), 72.1 (C-4′), 78.0 (C-5′), 62.7 (C-6′)] in the molecule. The value of the coupling constant ($J = 7.5$ Hz) of the signal for the anomeric hydrogen (δ_H 4.48) established a β-configuration. On the other hand, the chemical shift values of C-3 (δ_C 93.8) and C-2 (δ_C 71.5) indicated that the sugar moiety was linked to the cucurbitacin aglycone through the hydroxyl group at C-3. Furthermore, the HMBC correlation between C-3 and the anomeric hydrogen is consistent with this proposal. On the basis of these considerations, compound **1** was characterized as 25-*O*-acetyl-3-*O*-β-D-glucopyranosyl-23,24-dihydrocucurbitacin F.

The long term antihyperglycemic effect of the three extracts (*E. caribaeum*, CM and *H. latiflora*) and pure compounds **2**–**6** and **8**, using a classical chronic experiment with STZ-induced diabetic rats (Verspohl, 2002; Subash-Babu and Mainzen-Prince, 2004; Sezick et al., 2005; Jong-Yuh and Mei-Fen, 2005), was next performed. The results demonstrated that daily oral administration of the extract (50 mg/kg each time) of *H. latiflora*, *H. standleyana* and compounds **2**–**6** and **8** (15 mg/kg each time), twice a day, for 30 days, induced a significantly more pronounced antihyperglycemic effect in the STZ-diabetic rats (Figs. 1 and 2). The extracts of CM and *E. caribaeum* were less efficient in decreasing blood glucose levels in diabetic rats (Fig. 1), and compound **8** was inactive (Fig. 2). Compounds **3** and **4** also restored blood glucose levels to near normal values at the end of the experiment [Fig. 2 (30 days)]. However, the extract of *H. latiflora* (Fig. 1) and compound **5** (Fig. 2) brought back normal blood glucose levels, and the effect was comparable to that of the glibenclamide used as a positive control (Verspohl, 2002; Subash-Babu and Mainzen-Prince, 2004). The treatments with *H. latiflora* extract, as well as those with compounds **2** and **6** (Guerrero-Analco et al., 2005), also prevented body weight loss in hyperglycemic rats (Table 3); however, the effect was higher than that of glibenclamide. *E. caribaeum* and CM extracts, on the other hand, did not prevent weight loss in diabetic rats. In our previous study on the extract of *H. standleyana* and compound **2** and **6**, we had found that they prevented body weight loss in diabetic rats (Guerrero-Analco et al., 2005); therefore, the inefficacy of the CM extract to prevent body weight loss could be attributed to the presence of *E. caribaeum*.

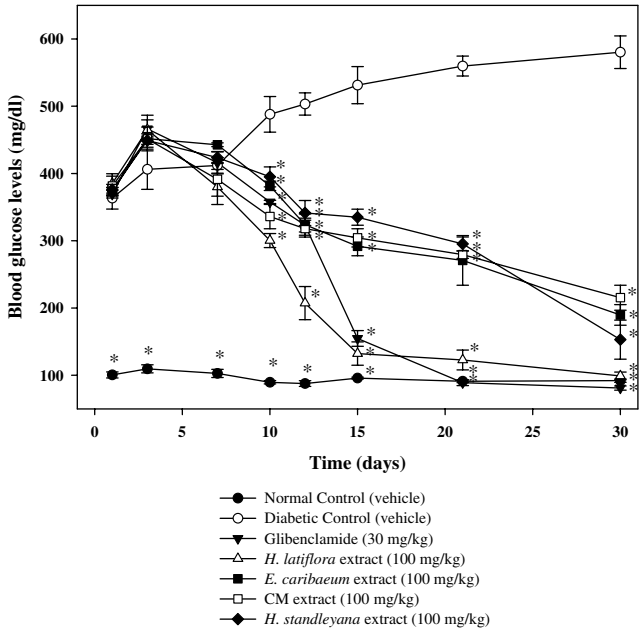
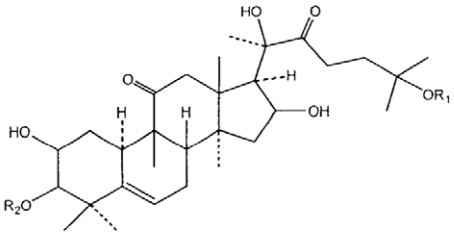


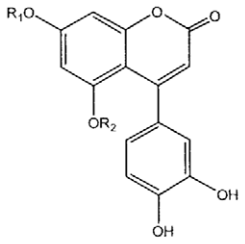
Fig. 1. Long-term effect of the extracts of *H. latiflora*, *E. caribeaum*, *H. standleyana*, CM (commercial mixture of copalchi) on blood glucose levels in STZ-diabetic rats. Each value is the mean ± SEM for six rats in each group. $^{*}p < 0.05$ significantly different ANOVA followed by Dunnett's *t*-test for comparison with the diabetic control group at same time.



1 R_1 = Ac R_2 = -β-D-glucopyranosyl

1a R_1 = Ac R_2 = H

2 R_1 = H R_2 = -β-D-glucopyranosyl



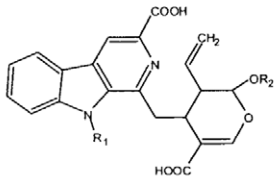
3 R_1 = Me R_2 = -β-D-glucopyranosyl

4 R_1 = Me R_2 = -β-D-galactopyranosyl

5 R_1 = H R_2 = -β-D-glucopyranosyl

6 R_1 = Me R_2 = -[β-D-apiofuranosyl-(1→6)-β-D-glucopyranosyl]

7 R_1 = Me R_2 = -[β-D-xylopyranosyl-(1→6)-β-D-glucopyranosyl]



8 R_1 = H R_2 = -β-D-glucopyranosyl

Comparison of the antihyperglycemic activity of compounds **3**–**5** established that the most active compound possesses a free hydroxyl group at C-7 in the 4-phenylcoumarin core. On the other hand, the comparison of the activity of compounds **3** and **4** indicates that the nature of the sugar moiety (glucose vs. galactose) does not influence biological activity.

One of the possible mechanisms by which the copalchis extracts and active principles exert their antihyperglycemic action in rats could be due to an insulin-release stimulatory

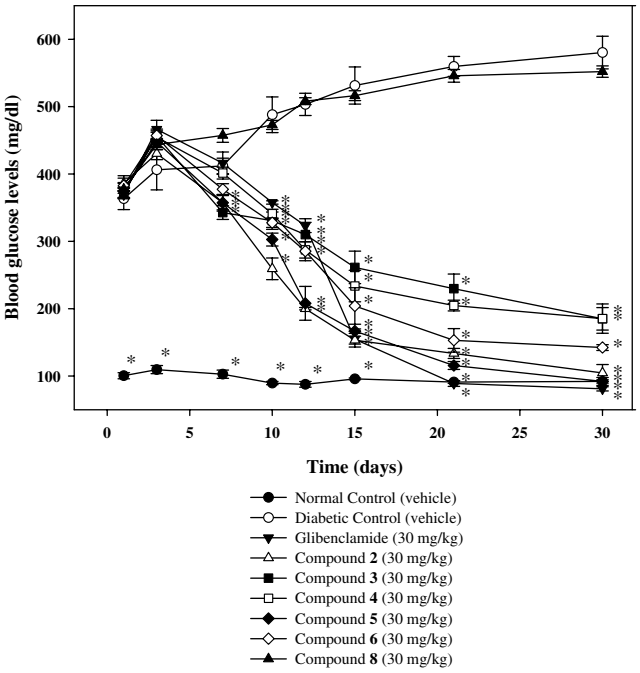


Fig. 2. Long-term effect of compounds **2**–**6** on blood glucose levels in STZ-diabetic rats. Each value is the mean ± SEM for six rats in each group. $^{*}p < 0.05$ significantly different ANOVA followed by Dunnett's *t*-test for comparison with the diabetic control group at same time.

effect; therefore, their action on plasma insulin on STZ-induced diabetic rats was investigated. This model was used because the induction of diabetes with a lower dose of STZ (50 mg/kg) produced an incomplete destruction of pancreatic β-cells even though the rats become permanently diabetic (Subash-Babu and Mainzen-Prince, 2004). Thus, after treatment with 50 mg/kg of STZ there should be some surviving β-cells. Furthermore, with some treatments their regeneration is also possible. The results summarized in Fig. 3 clearly demonstrate that the extracts of *H. latiflora* and *H. standleyana* as well as compounds **2**, **5** and **6** induced the secretion of insulin in a similar way to the positive control glibenclamide. Given that the treatments induced an increment in plasma insulin levels, they might increase the renewal of β-cells in the pancreas or permit the recovery of partially destroyed β-cells and stimulates pancreatic insulin secretion (Subash-Babu and Mainzen-Prince, 2004). Since the treatments with the *Hintonia* extracts and compounds **2**, **5** and **6** did not restore to normal values the insulin levels, it is highly possible that they exert their antihyperglycemic through an insulinomimetic mode of action (Verspohl, 2002).

The extract of *H. latiflora* significantly improved hepatic glycogen content in STZ-diabetic rats (Fig. 4) as well

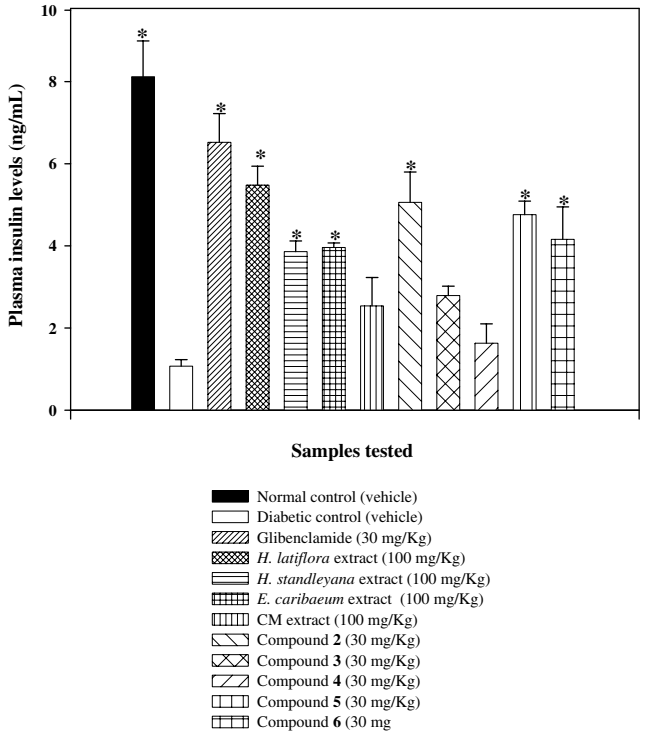


Fig. 3. Effect of the extracts of *H. latiflora*, *H. standleyana*, *E. caribaeum*, CM (commercial mixture of copalchi), and compounds **2**–**6** on plasma insulin levels in STZ-diabetic rats. Each value is the mean ± SEM for six rats in each group. $^{*}p < 0.05$ significantly different ANOVA followed by Dunnett's *t*-test vs. the diabetic control group at same time.

Table 3
Long-term effect of the extracts of *H. latiflora*, *E. caribeaum*, CM (commercial mixture of copalchi) and compounds **3**–**5** and **8** on body weight, in STZ-diabetic rats

Test samples	Body weight (g)				
	Dose (*per os*) mg/kg of bw	1st day	7th day	15th day	30th day
Normal control (vehicle)	–	213.4 ± 3.9*	249.9 ± 3.0*	274.6 ± 4.2*	288.2 ± 5.5*
Diabetic Control (vehicle)	–	169.8 ± 5.2	216.2 ± 5.2	194.8 ± 6.0	204.2 ± 9.2
Glibenclamide	30	178.3 ± 7.8	210.2 ± 4.1	212.8 ± 4.9	216.3 ± 8.9
Extract of *H. latiflora*	100	182.9 ± 7.7	227.2 ± 8.0	238.3 ± 12.4*	248.3 ± 16.4*
Extract of *E. caribeaum*	100	177.9 ± 10.6	213.4 ± 11.9	223.3 ± 13.2	217.7 ± 17.6
Extract of CM	100	173.3 ± 9.2	214.9 ± 5.9	220.8 ± 15.3	223.1 ± 20.4
Compound **3**	30	169.7 ± 4.8	213.3 ± 6.6	219.2 ± 8.1	230.3 ± 10.0*
Compound **4**	30	169.6 ± 11.6	205.9 ± 8.9	211.3 ± 12.0	221.3 ± 14.6
Compound **5**	30	190.3 ± 6.6	218.8 ± 8.7	225.2 ± 12.0*	241.3 ± 12.3*
Compound **8**	30	170.3 ± 6.6	188.8 ± 7.8	205.2 ± 11.0	201.3 ± 13.2

Each value is the mean ± SEM for six rats in each group.
* $p < 0.05$ significantly different ANOVA followed by Dunnett's *t*-test for comparison with the diabetic control group at same time.

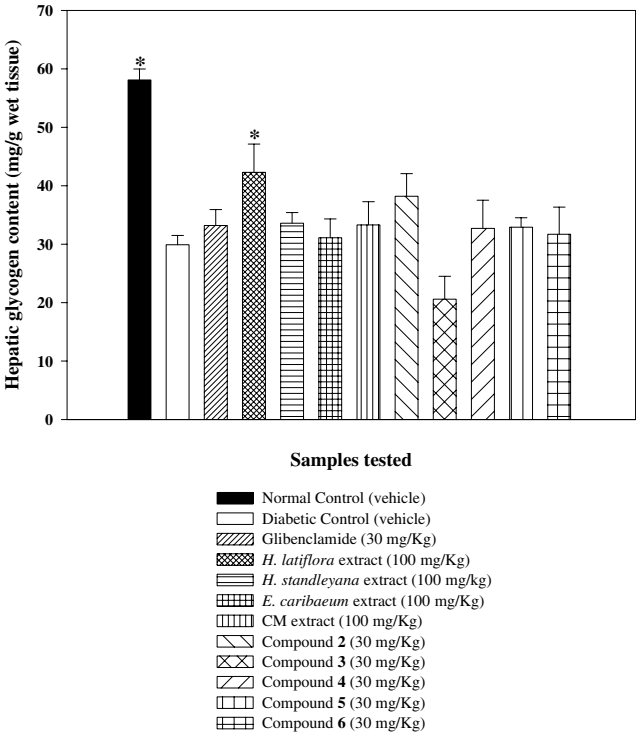


Fig. 4. Effect of the extracts of *H. latiflora*, *H. standleyana*, *E. caribaeum*, CM (commercial mixture of copalchi), and compounds **2**–**6** on glycogen hepatic content in STZ-diabetic rats. Each value is the mean ± SEM for six rats in each group. $^{*}p < 0.05$ significantly different ANOVA followed by Dunnett's *t*-test vs. the diabetic control group at same time.

suggesting that it might modulate glycogen breakdown by inhibiting glucose-6-phosphatase (G6Pase), a crucial enzyme for the final step of gluconeogenesis or glycogenolysis. Several antidiabetic agents, such as metformin and *p*-methoxycinnamic acid, a natural product, also inhibit glucose production by suppressing G6Pase activity. Alternatively, the extract could induce an increment in glucokinase activity in diabetic rats, which in turn regulated glycogen metabolism (Adisakwattana et al., 2005).

3. Conclusions

Our results corroborated further that the active principles of the Mexican copalchis of the Rubiaceae family are both 4-phenylcoumarins glycosides and cucurbitacins. This is thus the first report describing the antidiabetic principles of *H. latiflora* and beyond any doubt answers any questions raised by many researchers concerning the nature of the active principles of this Mexican plant species. The extract of *H. latiflora* and compound **5** were also the most effective in regulating blood glucose levels in hyperglycemic conditions, using both short and long term assays; their mode of action involves stimulatory effect on insulin secretion by β-cells in the pancreas. *H. latiflora* extract also stimulate hepatic glycogen metabolism. As for *H. standleyana*, *H. latiflora* biosynthesizes indole-monoterpenoid type alkaloids. The lack of efficacy of *E. caribaeum*, as well as the toxic effects previously demonstrated, should cause Mexican Health Authorities to alert local consumers about the risks in using this plant.

4. Experimental

4.1. General experimental procedures

Melting points were determined on a Fisher–Johns apparatus and are uncorrected. IR spectra were recorded as KBr pellets or film on a Perkin–Elmer 59913 spectrophotometer. Optical rotations were registered on a Perkin–Elmer 241 digital polarimeter. NMR spectra were recorded on a Varian VXR-300S spectrometer in CD_3OD, either at 300 MHz (^1H) or 75 (^{13}C) MHz, using tetramethylsilane (TMS) as an internal standard. EIMS were obtained on a JMS-AX505HA mass spectrometer, whereas positive FABMS data were obtained using a JEOL SX 102 mass spectrometer with a NBA matrix. Open column chromatography (CC) was carried out on silica gel 60 (70–230 mesh, Merck). TLC analyses were performed on silica gel 60 F_{254} plates (Merck), and visualization of the plates was carried out using a ceric sulfate (10%) solution in H_2SO_4 or anisaldehyde-H_2SO_4 reagent (for sugars).

4.2. Plant material

The stem bark of *H. latiflora* was collected in Chihuahua in June 2003 and identified by Dr. Robert Bye (Jardín Botanico, UNAM). A voucher specimen (Bye No 31, 735) is deposited at the National Herbarium (MEXU), Mexico City. The stem bark of *H. standleyana* Bullock was collected in Atenango del Rio, Guerrero, in January 2003 and identified by Celso Guerrero and Francisco Ramos from the National Herbarium (MEXU); a voucher specimen (P. Hersch No 824) is deposited at the ASFM-INAH Herbarium, Cuernavaca. The stem bark of *E. caribeaum* was collected in Atenango del Rio, Guerrero, in January 2003 and identified by Celso Guerrero and Francisco Ramos from the National Herbarium (MEXU). A voucher specimen (Marchena 1167098) is deposited at the ASFM-INAH Herbarium, Cuernavaca, State of Morelos. A commercial sample (CM) of "copalchi" composed by the stem barks of *H. standleyana* and *E. caribaeum* in a 1:4 ratio was purchased from the popular market of Sonora in Mexico City.

4.3. Extraction of stem bark of H. latiflora and isolation of compounds 1–8

The air-dried and pulverized material (3.0 kg) was macerated with CH_2Cl_2–MeOH (6 l × 3, 1:1) at room temperature for a duration of 6 days, with subsequent evaporation of the solvent in vacuo to afford a crude extract (160 g). An aliquot of this (115 g) was then dissolved in a minimal amount of MeOH, and from this solution compound **4**

(36 g) crystallized, m.p. 230 °C (lit m.p. 228–231 °C; Mata et al., 1987). The mother liquors were next subjected to silica gel (1.0 kg) CC in a glass column using $CHCl_3$ with increasing amounts of MeOH as eluents to yield 12 primary fractions (FH1-FH12). Fraction FH2 (2 g), eluted with $CHCl_3$–MeOH (92:8), was further applied to a second silica gel column using $CHCl_3$–EtOAc–MeOH (47.5:47.5:5) as a mobile phase to give nine fractions (FH2I–FH2IX). From fraction FH2III, compound **1** (200 mg) spontaneously crystallized. From fraction FH2V, compound **2** (1.0 g) crystallized, m.p. 201 °C (lit m.p. 199–201 °C; Mata et al., 1990). From fraction FH5, eluted with $CHCl_3$–MeOH (9:1), compound **3** (4.5 g), m.p. 238 °C (lit m.p. 237–238 °C; Mata et al., 1988). Fraction FH9 eluted with $CHCl_3$–MeOH (85:15), yielded compound **5** (6.5 g), m.p. 251 °C (lit m.p. 250–252 °C; Mata et al., 1990). Column chromatography over silica gel (600 g) of fraction FH10 (15 g), eluted with $CHCl_3$–MeOH (75:25), using $CHCl_3$–MeOH (9.5:0.5 → 0:1) as mobile phase yielded eight secondary fractions, FH10I-FH10VIII. From fraction FH10IV (1 g), eluted with $CHCl_3$–MeOH (8:2), compound **6** (100 mg), m.p. > 300 °C (lit m.p. > 300 °C; Guerrero-Analco et al., 2005) was obtained by crystallization ($CHCl_3$–MeOH 8:2). From fraction FH10VI (3.6 g) eluted with $CHCl_3$–MeOH (7:3), compound **7** (468 mg) crystallized. Finally, column chromatography over silica gel (100 g) of FH12 (2 g), eluted with $CHCl_3$–MeOH (6:4), using CH_2Cl_2–MeOH (8:2 → 0:1) as mobile phase yielded five secondary fractions, FH12I-FH12V. From fraction FH12III (10 g), eluted with $CHCl_3$–MeOH (4:6), compound **8** (1 g) crystallized, m.p. > 300 °C (lit m.p. > 300 °C; Déciga-Campos et al., 2006).

4.4. Extraction of 25-O-acetyl-3-O-β-D-glucopyranosyl-23,24-dihydrocucurbitacin F (1) from the stem bark of H. standleyana

Dried and shredded stem bark (300 g) was macerated with CH_2Cl_2–MeOH (2 l × 3, 1:1) for 6 days at room temperature. The combined extracts were evaporated in vacuo to yield a brown residue (80 g), which was applied to a glass column packed with silica gel (800 g). This was next eluted with hexane–EtOAc (1:1 → 0:1) and EtOAc–MeOH (1:0 → 0:1) to give five fractions (FS1–FS5). Fraction FS2 (2.0 g), eluted with EtOAc–MeOH (9:1), was further subjected to silica gel CC using $CHCl_3$–EtOAc–MeOH (47.5:47.5:5) as mobile phase to give 10 fractions (FS4I–FS4X). From fraction FH4V, compound **1** (30 mg) spontaneously crystallized; from fraction FH4X precipitated compound **2** (120 mg), m.p. 201–203 °C (lit m.p. 199–201 °C; Guerrero-Analco et al., 2005).

4.5. 25-O-Acetyl-3-O-β-D-glucopyranosyl-23,24-dihydrocucurbitacin F (1)

Glassy white solid; m.p. 158–161 °C, $[\alpha]_D + 45 (c = 0.2$, MeOH); IR ν_{max} (KBr) cm^{-1}: 3400, 2920, 1710, 1690, 1630, 1455, 1430 and 1255; HRFABMS: $m/z = 725.8731$ $[M+H]^+$ (calcd. for $C_{38}H_{60}O_{13}$: 725.8752); FABMS: $m/z = 748$ $[M+Na+H]^+$; 665 $[M–CH_3CO_2H+H]^+$; 563 $[M–162+H]^+$. NMR-Data [^{13}C (75.5 MHz, $CDOD_3$)/^1H (300 MHz, $CDOD_3$)]: C-1 [δ_C 34.1/δ_H 1.78 (1H, *m*), 2.23 (1H, *m*)], C-2 [δ_C 71.5/δ_H 3.84 (1H, *m*)], C-3 [δ_C 93.8/δ_H 2.99 (1H, *d*, $J = 9$ Hz)], C-4 (δ_C 43.3), C-5 (δ_C 142.5), C-6 [δ_C 120.5/δ_H 5.78 (1H, *bd*, $J = 5.4$ Hz)], C-7 [δ_C 25.5/δ_H 1.21 (1H, *dd*, $J = 13.2$, 2.1 Hz), 1.37 (1H, *m*)], C-8 [δ_C 44.3/δ_H 1.93 (1H, *m*)], C-9 (δ_C 49.1), C-10 [δ_C 34.8/δ_H 2.46 (1H, *m*)], C-11 (δ_C 216.0), C-12 [δ_C 49.8/δ_H 3.22 (2H, *m*)], C-13 (δ_C 48.7), C-14 (51.8), C-15 [δ_C 46.7/δ_H 1.93 (2H, *m*)], C-16 [δ_C 71.5/δ_H 3.30 (1H, *m*)], C-17 [δ_C 59.5/δ_H 2.52 (1H, *d*, $J = 7.2$ Hz)], C-18 [δ_C 20.5/δ_H 0.88 (3H, *s*)], C-19 [δ_C 19.7/δ_H 1.36 (3H, *s*)], C-20 (δ_C 80.9), C-21 [δ_C 25.6/δ_H 1.28 (3H, *s*)], C-22 (δ_C 216.6), C-23 [δ_C 32.8/δ_H 1.58 (2H, *m*)], C-24 [δ_C 35.8/δ_H 2.02 (2H, *m*)], C-25 (δ_C 83.1), C-26 [δ_C 26.2/δ_H 1.44 (3H, *s*)], C-27 [δ_C 26.2/δ_H 1.44 (3H, *s*)], C-28 [δ_C 23.4/δ_H 0.99 (3H, *s*)], C-29 [δ_C 24.8/δ_H 1.28 (3H, *s*)], C-30 [δ_C 20.4/δ_H 1.1 (3H, *s*)], C-1′ [δ_C 106.8/δ_H 4.48 (1H, *d*, $J = 7.5$ Hz)], C-2′ [δ_C 76.2/δ_H 3.30 (1H, *m*)], C-3′ [δ_C 78.5/δ_H 3.34 (1H, *m*)], C-4′ [δ_C 72.1/δ_H 3.32 (1H, *m*)], C-5′ [δ_C 78.0/δ_H 3.22 (1H, *m*)], C-6′ [δ_C 62.7/δ_H 3.69 (1H, *dd*, $J = 11.7$,5.1 Hz), 4.06 (1H, *m*)], CH_3-C=O [δ_C 22.3/δ_H 1.95 (3H, *s*)], $CH_3 - C = O$ (δ_C 172.4).

4.6. Acid hydrolysis of 1

Compound **1** (5 mg), dissolved in 2 M HCl (10 ml) was heated until reflux began, this being held for 2 h. A white powder precipitated from the acid solution and after usual work-up, aglycone **1a** (2 mg) was obtained (Reguero et al., 1987); glucose was identified by TLC following comparison with an authentic sample.

4.7. Extraction of the stem bark of E. caribeaum and CM (commercial mixture of copalchi)

Dried and shredded stem bark (250 g) of *E. caribeaum* was macerated with CH_2Cl_2–MeOH (6 l × 3, 1:1) for 6 days at room temperature. The combined extracts were evaporated in vacuo to give a light brown residue (80 g). Dried and shredded stem bark (300 g) of CM was macerated with CH_2Cl_2–MeOH (1:1) (6 l × 3) for 6 days at room temperature. The combined extracts were evaporated in vacuo to yield a brown residue (90 g).

4.8. Biological assays

4.8.1. Test animals

Male Wistar normoglycemic rats, 55–60-day-old, weight 180–220 g, were obtained from Centro UNAM-Harlan (Harlan México, SA de CV). Procedures involving animals and their care were conducted in conformity with the Mexican Official Norm for Animal Care and Handing (NOM-062-ZOO-1999) and in compliance with international rules

on care and use of laboratory animals. The animals were housed under standard laboratory conditions and maintained on a standard pellet diet and water *ad libitum*.

4.8.2. Preparation of the test samples

Samples were suspended in 0.05% Tween® 80 in saline solution. Glibenclamide (Sigma Co., St. Louis, MO, USA) was used as a hypoglycemic model drug (Verspohl, 2002). Control rats group only received the vehicle (0.05% Tween® 80 in saline solution) in the same volume (0.5 ml of vehicle/100 g of bw) by the same route.

4.8.3. Collection of blood samples and determination of blood glucose levels

Blood samples were collected from the caudal vein by means of a little incision at the end of the tail. Blood glucose levels (mg/dl) were estimated by enzymatic glucose oxidase method using a commercial glucometer (One Touch Ultra, Jonhson–Jonhson, California, USA). The percentage variation of glycemia for each group was calculated with respect to initial (0 h) level according to:

$$\%\ \text{Variation of glycemia} = [(G_i - G_t)/G_i] \times 100\%$$

where G_i is initial glycemia values and G_t is the glycemia value after samples administration.

4.8.4. Acute hypoglycemic assay

Normal rats were placed in single cages with wire-net floors and deprived of food for 12 h before experimentation but allowed free access to tap water throughout. The crude extracts (at the dose of 10, 30, 100 and 300 mg/kg of bw) of *H. latiflora* and *E. caribaeum* were suspended in 0.05% Tween® 80 in saline solution. Glibenclamide (10 mg/kg of bw) was suspended in the same vehicle. The samples were freshly prepared immediately before experimentation and administered by the intragastrical route. Control rats received only the vehicle (0.05% Tween® 80 in saline solution) by the same route. Blood samples were collected at 0, 1.5, 3, 5, 7, 9 h after drugs administration.

4.8.5. Effect on diabetic animals (non-insulin dependent diabetes model-NIDDM)

4.8.5.1. Induction of experimental diabetes. Diabetes mellitus type 2 was induced in rats by a single intraperitoneal injection of freshly prepared STZ (50 mg/kg; Sigma. St. Louis, MO, USA) dissolved in 0.1 M citrate buffer, pH 4.5, in a volume of 1 ml/kg of bw. After 7 days of STZ administration, blood glucose levels of each rat were determined. Rats with blood glucose levels higher than 250 mg/dl were considered diabetic and were included in the study (Verspohl, 2002; Guerrero-Analco et al., 2005).

4.8.5.2. Acute antihyperglycemic assay. Diabetic rats were treated exactly as the normal rats (see Section 4.8.4).

4.8.6. Effects of daily treatment with crude extracts and pure compounds in STZ-induced diabetic rats

DM was induced in 66 rats by the same method showed in the section of the induction of experimental diabetes. Thereafter, the extracts, pure compounds and glibenclamide were administered twice (9 a.m. and 6 p.m.) on a daily basis, for a period of 30 days, to STZ-diabetic rats which were divided into eight groups (A–H). A nine group of normal rats (Group I) was used as a control. Groups H (diabetic control) and I received vehicle (0.05% Tween® 80 in saline solution) *per os* during 30 days. Group A received glibenclamide (15 mg/kg; 30 mg/kg daily). Groups B to G received different treatments: B, *H. latiflora* extract (50 mg/kg; 100 mg/kg daily); C, *E. caribaeum* extract (50 mg/kg; 100 mg/kg daily); D, CM of "copalchi" (50 mg/kg; 100 mg/kg daily); E, compound **3** (15 mg/kg; 30 mg/kg daily); F, compound **4** (15 mg/kg; 30 mg/kg daily) and G, compound **5** (15 mg/kg; 30 mg/kg daily). Compounds **2**, **6** (15 mg/kg; 30 mg/kg daily) and *H. standleyana* extract (50 mg/kg; 100 mg/kg daily) were also included in the experiments for comparative purposes. All tested materials were suspended in the same vehicle and administered *per os* daily for 30 days. The animals were housed under standard laboratory conditions and maintained with free access to water and food during all the experiment. Blood glucose concentration on both diabetic (Groups A–H) and control group (I) animals was estimated at days 0, 3, 7, 10, 12, 15, 21 and 30. The body weights of the animals were measured concomitantly to the blood glucose analyses. All experiments were carried out using six animals per group.

4.8.7. Determination of hepatic glycogen and plasma insulin levels

After 30 days of treatment with any of the extracts (*H. stadleyana*; *H. latiflora*; *E. caribaeum* and CM) or tested compounds (**2**–**6**; **8**), the animals (normal and diabetic) were decapitated after an overnight fast. Blood was collected in heparinized tubes and plasma was separated after centrifugation. Liver tissues were excised immediately and stored in ice-cold containers. Hepatic glycogen content was measured according to Murat and Serfaty (1974). Briefly, liver samples (100 mg) were homogenized in 2 ml of ice-cold citrate buffer (0.1 M, pH 4.2). Immediately after homogenization, 10 μl of the sample was used to determine free glucose in the tissue. Amyloglucosidase (exo-1,4-α-glucosidase, EC 3.2.1.3, Cat #A9228, Sigma Chemical, Co, St Louis, MO, USA) dry powder was then mixed with the homogenate (1 mg/ml) and allowed to stand at room temperature for 2 h. A 10 μl sample was then used to determine total glucose amounts. Initial free glucose values were subtracted, with the difference being the value used to calculate the glycogen content in the tissue. Glucose was determined using a commercial kit (Glucose TR, Cat 1001190, Spinreact, Girona, Spain). Plasma insulin levels were estimated by the Rat Insulin Enzyme Immunoassay Method using

a SpiBio Kit (Microplate Reader BIO-RAD Model 680) (Grassi and Pradelles, 1991).

4.9. Statistical analysis

Data are expressed as the means ± SEM for the number ($n = 6$) of animals in each group. Repeated measurement analysis of variance (ANOVA) was used to analyze the changes in blood glucose and other parameters. Dunnett range post-hoc comparisons were used to determine the source of significant differences where appropriate; ($p < 0.05$) was considered statistically significant. Sigma plot and Sigma Stat (version 9.0) software were used for statistics and plotting.

Acknowledgements

This work was supported by Grants of Consejo Nacional de Ciencia y Tecnología (CONACyT, Proyecto C01-018) and DGAPA-UNAM IN212005. We are indebted to Alejandro Urzua, Victor Lemus and Chabetty Vargas for their collaboration in this work. The technical assistance of Isabel Rivero-Cruz, Laura Acevedo, Rosa del Villar, Marisela Gutiérrez and Georgina Duarte-Lisci is also recognized. J.A. Guerrero-Analco acknowledges fellowships from CONACyT and Dirección General de Estudios de Posgrado, UNAM, to support graduate studies.

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