Short communication

Determination of bioactive markers in *Cleome droserifolia* using cell-based bioassays for antidiabetic activity and isolation of two novel active compounds

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**ABSTRACT**

The antidiabetic activities of the aqueous (AqEx) and ethanolic (AlEx) extracts of *Cleome droserifolia* (Forsk.) Del., were tested in cultured C2C12 skeletal muscle cells and 3T3-L1 adipocytes. An 18-h treatment with the AqEx increased basal glucose uptake by 33% [insulin equivalent (IE) = 1.3 ± 0.04] in muscle cells comparable to a 25.5% increase caused by 100 nM insulin (IE = 1 ± 0.03). Fractionation of the tested AqEx yielded hexane (HxFr), chloroform (ClFr) and ethyl acetate (EtFr) fractions which exerted 38, 52 and 35% increase in the glucose uptake corresponding to an IE of 1.5 ± 0.06, 2.0 ± 0.04 and 1.4 ± 0.04, respectively. Only the ClFr and EtFr accelerated the triglyceride accumulation [rosiglitazone equivalent (RE) was 0.9 ± 0.13 and 0.63 ± 0.12, respectively] in pre-adipocytes undergoing differentiation comparable with 10 μM rosiglitazone. Six terpenoids (C1–C6) and three flavonol glycosides (F1–F3) were isolated from the active ClFr and EtFr, respectively, and identified. C5, C2 and C4 had an IE of 0.86 ± 0.05, 1.01 ± 0.04 and 0.9 ± 0.08, while F1, F2 and F3 gave an IE of 1.3 ± 0.05, 2.3 ± 0.05 and 2.0 ± 0.04, respectively. We could conclude that the reported antihyperglycemic activity of *Cleome droserifolia* is attributed to significant insulin-like effects in peripheral tissues, and that compounds F2 and F3, being highly active, could be used as bioactive markers to standardize the *C. droserifolia* herbal extract.

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**Introduction**

Herbal remedies that stem from Egyptian traditional medicine hold a great promise against type II diabetes. However, ensuring the quality of these herbal products and developing quality control tools remain big challenges. One promising avenue involves cell-based bioassays that can be used to identify active principles; these then represent bioactive markers that can be used to standardize herbal preparations and hence ensure their quality.

The dried herb of *Cleome droserifolia* (Forsk.) Del., is a plant of the Cleomaceae family (El-Askary 2005). Its decoction is widely used by the Bedouins of the southern Sinai for the treatment of diabetes (Abdel-Hady 1998). The antihyperglycemic activities of different extracts of this herb have been validated by several studies (Abdel-Hady 1998; Abdel-Kawy et al. 2000; Mohamed et al. 1997; Nicola et al. 1996). Nevertheless, the target tissues and mechanisms of action of this herb are not yet well understood and the antidiabetic activity has not been correlated with any of its active ingredients.

We decided to carry out a biologically guided fractionation for *Cleome droserifolia* aerial parts with the aim of defining target tissues and the corresponding bioactive compounds through which the extract of this plant exerts its antihyperglycemic activity. To this aim, we used bioassays involving C2C12 myotubes and 3T3-L1 adipocytes.

**Materials and methods**

**Plant material**

The aerial parts of *Cleome droserifolia* were collected from wildly growing populations on El-Ketar mountain, Hurghada, Egypt and were authenticated by Prof. Dr. M. Gebali (Plant Taxonomy and Egyptian Flora Department, National Research Center, Giza, Egypt). They were dried, powdered and extracted by percolation with distilled water or 95% ethanol. The dried aqueous extract was suspended in distilled water and partitioned successively using n-hexane, chloroform, ethyl acetate and n-butanol saturated with water. ClFr and EtFr were further fractionated for the isolation of compounds (C1–C6) and (F1–F3), respectively (details of isolation and structural elucidation of these compounds will be published separately). Dried extracts, fractions and isolated compounds were dissolved in 0.1% DMSO for the biological screening.
HPLC analysis for active fractions

An HPLC method was developed for the analysis of the active ClFr and EtFr. An Agilent Technologies 1100 series HPLC was used, equipped with an Agilent 1200 series G1322A quaternary pump and degasser, and a G1314A variable wavelength detector. Isolated compounds and corresponding fractions were injected into a Lichrosphere 100 RP-18, 5 μm, 250 × 4 mm column (Merck, Germany), guarded by a 5 μm, 10 × 4 mm guard column. The mobile phases used were methanol (solvent A) and 0.3% orthophosphoric acid in water (solvent B) for the ClFr, and acetonitrile (solvent C) and solvent B for the EtFr. Gradient elution was carried out at a flow rate of 1.0 ml/min (0–16 min 65 to 75% A in B, 16–25 min 75 to 100% A in B, 25–30 min 100% A in B for ClFr and 0–25 min 10 to 75% C in B for EtFr). The injection volume was 20 μl and UV detection at 230 and 325 nm, respectively.

Cell culture

C2C12 myoblasts and 3T3-L1 preadipocytes were cultured in Dulbecco’s modified Eagle’s medium (DMEM) as previously described (Benhaddou-Andaloussi et al. 2008, 2010). Cells were treated with 200 μg/ml of extracts and fractions, and 50 μM of the isolated compounds. Only 100 μg/ml of the extracts were applied for treating the 3T3-L1 preadipocytes. These concentrations were based on pre-determined maximum non-toxic concentrations in the employed cell lines following 18-h treatment.

Glucose transport assay

Differentiated C2C12 cells grown in 12-well culture plates were treated with the tested solutions, vehicle control (0.1% DMSO) and a positive control [400 μg/ml metformine] for 18 h. Insulin 100 nM was added to one well as a second positive control during the assay (Benhaddou-Andaloussi et al. 2008; Spoor et al. 2006). Three to five independent experiments, each of three replicates, were performed.

Adipogenesis assay

3T3-L1 cells seeded in 12-well plates were treated with the tested solutions or 0.1% DMSO vehicle in differentiation medium during the entire differentiation period as described before (Benhaddou-Andaloussi et al. 2008; Spoor et al. 2006). Rosiglitazone 10 μM (Alexis Biochemicals, San Diego, CA, USA), dissolved in DMSO, was used as a positive control. Three separate experiments, each of three replicates, were performed. The mean value obtained from the negative control was considered as the background and subtracted from all other readings.

Statistical analysis

All reported data are the mean ± SEM of the indicated number of experiments. Results were analysed by one-way analysis of variance (ANOVA) and Fisher post hoc test using StatView software (SAS Institute Inc, Cary, NC, USA). Statistical significance was set at p ≤ 0.05 or p ≤ 0.0001.

Results and discussion

HPLC fingerprint chromatograms

An HPLC method was developed to analyse the active terpene (ClFr) and flavonoid (EtFr) containing fractions. Isolated terpenoidal and flavonoidal compounds were dissolved in methylene chloride and methanol (HPLC grade, Sigma–Aldrich), respectively, and injected separately. The retention time of each compound was determined. The ClFr (23 mg/ml methylene chloride) and EtFr (2 mg/ml methanol) were then analysed and the corresponding isolated compounds were identified in each chromatogram (Fig. 2A and B).

Cell-based bioassays for antidiabetic activity

Enhancement of glucose uptake in muscle cells

Differentiated C2C12 myotubes are insulin responsive and GLUT4 containing cells (He et al. 2007). An 18-h treatment with the AgEx caused a 33% increase in the basal glucose uptake which is similar to that induced by 100 nM insulin, while the AlEx showed no effect at all (Fig. 1A). Treating the cells for the same period of time revealed ClFr to be the most active among the AgEx subfractions. It gave an IE of 2.0 ± 0.04, which is 2 times the effect of 100 nM insulin (supraphysiologic dose) (Fig. 1A). This agrees with the previously reported in vivo data stating that only the chloroform and the total aqueous extracts of C. drossifolia revealed a decrease in blood sugar level in rats (Ismael 1992). The rank order of various solvent extracts exerting antihyperglycemic action (reduction of glucose level back to normal levels) in diabetic male rats was found to be the highest with benzene (which possesses a relatively low dielectric constant like chloroform), followed by ethyl acetate, n-hexane, and finally alcohol extracts of C. drossifolia (Abdel-Kawy et al. 2000).
Chromatogram of guai-10 at 40 triglyceride.Acceleration retention compounds: C. 100 C4 100 27.4, C3 C2 0.08, C2 100 C1 E, C1 and C2 28.5, C4 3T3-L1 and flavonol this extract. To compared to other the basal uptake 60 and 53%, corresponding to 2.3 and 2 times the effect of insulin, respectively. These activities were substantially larger than the parent EtFr indicating an enrichment of pharmacological action. Compound F1 had an IE of 1.3 ± 0.05 (Fig. 1A), which was close to the activity of the parent EtFr. Once again, future studies should address the possibility of additive or synergistic effects. Oral administration of 100 and 200 mg/kg of the methanolic extract of C. droserifolia (containing quercetin, kaempferol, isorhamnetin and three other phenolic acids) for 3 weeks significantly restored the blood glucose level in alloxan-induced diabetic rats to near the physiological values (El Naggar et al. 2005). The presence of methoxy groups could also contribute to the relatively higher activity of compounds F1, F2 and F3 (Nour et al. 2010).

Acceleration of adipogenesis in human cells
Rosiglitazone and other members of the thiazolidinedione (glitazone) family are known to increase insulin sensitivity in muscle and fat tissue (Konrad et al. 2005). To test for glitazone-like activity, the ability of increasing triglyceride accumulation was measured in differentiating 3T3-L1 adipocytes treated with the extracts throughout their differentiation period. Both ALEX and AQEX did not show a significant activity compared to control cells. In contrast, treatment with the CIFl fraction resulted in a 5-fold greater triglyceride content than in control cells, indicating a higher rate of adipogenesis comparable to that caused by 10 μM rosiglitazone. Only compounds C5 and C3 (a novel diterpene derivative) showed a significant effect, which amounted to about half that of rosiglitazone (RE of 0.52 ± 0.2 and 0.55 ± 0.22, respectively). This pattern of biological activity is similar to that obtained with glucose transport in C2C12 cells and again raises the question of additive or synergistic effects. The EtFr also showed a significant increase in triglyceride content (RE = 0.6 ± 0.12), as well as one of its isolates, F2 (RE = 0.4 ± 0.26) (Fig. 1B). F2 was thus again the most biologically active principle of the EtFr extract.

Conclusion
The CIFl and EtFr of C. droserifolia aqueous extract were found to have significant insulin-like effects in peripheral tissues, namely the stimulation of basal glucose uptake in skeletal muscle cells and glitazone-like enhancement of adipogenesis. Terpenes isolated from CIFl showed less activity than the total CIFl, while methoxy-substituted flavonoid glycosides (F2 and F3) were twice as active as the mother CIFl or insulin in stimulating glucose uptake in differentiated C2C12 myotubes. This indicates that muscle glucose uptake may participate in the glucose-lowering action of C. droserifolia and that some isolates (notably F2 and F3) may represent promising species for novel oral hypoglycemic drug development. This relates to the substantially greater activity of the compounds compared to the positive insulin control and will depend on their bioavailability and safety profiles as tested in appropriate in vivo and clinical settings. Isolates from both fractions were less than or as active as the mother fractions in enhancing adipogenesis in 3T3-L1 adipocytes. Nonetheless, these results raise the possibility that C. droserifolia or some of its components may act, at least in part, as do glitazones and in as powerful a manner as the rosiglitazone positive control. Structure-activity relationships for the bioactive compounds need further investigations to be understood.

Conflict of interest
No conflict to disclose.

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