Journal of Ethnopharmacology 127 (2010) 1–6

Contents lists available at ScienceDirect

Journal of Ethnopharmacology

journal homepage: www.elsevier.com/locate/jethpharm

The antidiabetic plants *Tecoma stans* (L.) Juss. ex Kunth (Bignoniaceae) and *Teucrium cubense* Jacq (Lamiaceae) induce the incorporation of glucose in insulin-sensitive and insulin-resistant murine and human adipocytes

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ARTICLE INFO

Article history:
Received 21 May 2009
Received in revised form 27 August 2009
Accepted 25 September 2009
Available online 8 October 2009

Keywords:
Tecoma stans
Teucrium cubense
3T3-F442A cells
Human preadipocytes
Adipogenesis
2-NBDG uptake

ABSTRACT

Aim of the study: Tecoma stans (L.) Juss. ex Kunth (Bignoniaceae) and *Teucrium cubense* Jacq (Lamiaceae) are plants extensively used for the empirical treatment of diabetes mellitus, but their antidiabetic mechanisms remain to be clarified. In this study, the effect of aqueous extracts of *Tecoma stans* (TSE) and *Teucrium cubense* (TCE) on the glucose uptake in adipose cells was evaluated.
Materials and methods: Non-toxic concentrations of TSE and TCE were assayed on the adipogenesis and 2-NBDglucose uptake in insulin-sensitive and insulin-resistant murine 3T3-F442A and human subcutaneous adipocytes.
Results: Both extracts stimulated 2-NBDG uptake by insulin-sensitive and insulin-resistant adipocytes in a concentration-dependent manner. In insulin-sensitive cells, TSE 70 μg/ml stimulated 2-NBDG uptake by 193% (murine) and by 115% (human), whereas the same concentration of TCE induced the 2-NBDG uptake by 112% (murine) and 54% (human). In insulin-resistant adipocytes, TSE induced the 2-NBDG uptake by 94% (murine) and 70% (human), compared with the incorporation shown by insulin-sensitive adipocytes stimulated by the hormone, whereas TCE induced the incorporation of 2-NBDG by 69% (murine) and 31% (human). On the other hand, TSE and TCE exerted only minimal or null proadipogenic effects on murine and human preadipocytes.
Conclusion: Tecoma stans and *Teucrium cubense* exert their antidiabetic effects stimulating glucose uptake in both insulin-sensitive and insulin-resistant murine and human adipocytes without significant proadipogenic or antiadipogenic side effects.

1. Introduction

Type 2 diabetes mellitus (T2-D) represents a serious public health problem since it accounts for substantial portions of national health expenditures worldwide (Balkrishnan et al., 2003; Arredondo et al., 2005). The undesirable side effects of drugs currently used for the treatment of T2-D (Cheng and Fantus, 2005) and the limited access to public health systems in low-income communities motivate patients to use alternative therapies. Medicinal plants constitute a common alternative treatment for T2-D in many parts of the world (Pagán and Tanguma, 2007). This is in accordance with estimations that 25% of modern medicines are derived from plants and the global market for herbal medicines currently stands at over US$ 60 billion annually and is growing steadily (WHO, 2009).

Tecoma stans (L.) Juss. ex Kunth (Bignoniaceae) and *Teucrium cubense* Jacq (Lamiaceae) are extensively employed in the Mexican traditional medicine for the treatment of T2-D. The hypoglycemic activity of *Tecoma stans* was confirmed in animal models (Román-Ramos et al., 1991) and the alkaloid tecomine was shown to stimulate the glucose uptake by adipocytes isolated from normoglycemic rats, although it did not reduce the plasma glycemia in *db*/*db* mice (Costantino et al., 2003). Recently, it was shown that an aqueous extract of *Tecoma stans* modulated the glucose intestinal absorption in rats (Aguilar-Santamaría et al., 2009), which nevertheless does not exclude the possibility of alternative antidiabetic

Abbreviations: 2-NBDG, 2-[N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl amino]-2-deoxy-D-glucose; CS, Calf serum; DMEM, Dulbecco's modified Eagle medium; FBS, Fetal bovine serum; hAM, Human adipogenic medium; hBM, Human basal medium; mAM, Murine adipogenic medium; mBM, Murine basal medium; NAM, Non-adipogenic medium; RGZ, Rosiglitazone; TSE, *Tecoma stans* aqueous extract; TCE, *Teucrium cubense* aqueous extract; TNF-α, Tumor necrosis factor alpha; T2-D, Type 2 diabetes.

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doi:10.1016/j.jep.2009.09.060

mechanisms in this plant. It has also been reported that aqueous extracts from *Teucrium cubense* decreased plasma glucose levels in healthy rabbits (Román-Ramos et al., 1991) although the mechanism of action was not determined. Therefore, in spite of the common use of *Tecoma stans* and *Teucrium cubense* as antidiabetics, the mechanisms mediating their hypoglycemic properties, as well as their active principles, remain to be fully understood.

Using the well-characterized murine preadipose 3T3-F442A cell line (Green and Kehinde, 1975) and human subcutaneous preadipocyte cultures (Herrera-Herrera et al., 2009), this work demonstrates that aqueous extracts of *Tecoma stans* (TSE) and *Teucrium cubense* (TCE) stimulate glucose uptake in insulin-sensitive and insulin-resistant murine and human adipocytes. This work also presents evidence that the antidiabetic extracts of *Tecoma stans* and *Teucrium cubense* do not exert appreciable proadipogenic effects on murine or human preadipocytes.

2. Materials and methods

2.1. Materials

Murine 3T3-F442A preadipocytes and adult cat serum were obtained from Dr. W. Kuri-Harcuch (CINVESTAV, México). Methods for obtaining normal human subcutaneous preadipocytes were previously described by Herrera-Herrera et al. (2009). Dulbecco's modified Eagle medium (DMEM), Leibovitz L15 medium (L15) and fetal bovine serum (FBS) were from GIBCO BRL (Grand Island, NY, USA) whereas calf serum (CS) was from HyClone (Logan, UT, USA). Human tumor necrosis factor alpha (TNF-α) and Rosiglitazone (RGZ) were obtained from Preprotech (London, UK) and Cayman Chem. (Ann Arbor, MI, USA), respectively, and 2-[N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl) amino]-2-deoxy-D-glucose (2-NBDG) was from Invitrogen (Carlsbad, CA, USA). All other chemicals were from Sigma Chem. (St. Louis, MO, USA). Standards used for the chemical characterization of extracts were all 95% purity or higher, according to the manufacturer.

2.2. Plant material and extraction

Leaves of *Tecoma stans* and leaves and branches of *Teucrium cubense* were collected in the municipality of Querétaro, México, and taxonomically validated by a specialist (M. Gómez-Sánchez). Voucher specimens of *Tecoma stans* (M. Gómez 700, QMEX) and *Teucrium cubense* (M. Gómez 701, QMEX) were preserved at the herbarium of Facultad de Ciencias Naturales, Universidad Autónoma de Querétaro (QMEX), México. Aqueous extracts of *Tecoma stans* (TSE) and *Teucrium cubense* (TCE) were obtained by boiling 50 g of raw material in one liter of sterile double-distilled water in a reflux condenser as described previously by Alonso-Castro and Salazar-Olivo (2008).

2.3. Chemical characterization of TSE and TCE

Total phenols in TSE and TCE were determined by the Folin-Ciocalteu assay (McDonald et al., 2001) with some modifications. Briefly, 0.25 ml of plant extract (0.1 mg/ml) were mixed with 2.5 ml of Folin-Ciocalteu reagent (diluted 1:10 with distilled water) and allowed to stand for 5 min at room temperature. Then, 2 ml of 1 M Na_2CO_3 were added and the mixtures incubated at room temperature for 2 h. Finally, total phenols were estimated at 765 nm using a spectrophotometer (Jenway 6405 UV/vis Dunmow, Essex, UK). A standard curve was prepared using gallic acid (0–250 mg/l). Total phenol values are expressed as gallic acid equivalents (GAE) g/kg of plant extract.

Total flavonoids in TSE and TCE were estimated using the Dowd method as adapted by Arvouet-Grand et al. (1994). Briefly, 2.5 ml of 2% $AlCl_3$ in methanol were mixed with 2.5 ml of plant extract (0.1 mg/ml). The mix was allowed to stand for 10 min at room temperature and the total flavonoid content was determined by spectrophotometry at 415 nm using a quercetin (0–250 mg/l) standard curve. Flavonoid contents are expressed as quercetin equivalents (QE) g/kg of plant extract.

Total alkaloid content in TSE and TCE was determined by the method proposed by Shamsa et al. (2008) with some modifications. Briefly, 0.5 ml of plant extract (0.1 mg/ml) was mixed with 2.5 ml of phosphate buffer solution (pH 4.7; prepared with 0.2 M sodium phosphate and 0.2 M citric acid) into a separatory funnel. Then, 2.5 ml of bromocresol green solution 0.1 mM and 5 ml of chloroform were added and solutions mixed vigorously. Finally, the water phase was separated and its total alkaloid content was determined by spectrophotometry at 470 nm using an atropine (0–120 mg/l) standard curve. Alkaloid values are expressed as atropine equivalents (AE) g/kg of plant extract.

2.4. Determination of TSE and TCE non-toxic concentrations for bioassays

3T3-F442A preadipocytes were seeded (1×10^4 cells/well) in 24-well plates (Corning Glass Works, Corning, NY, USA) with DMEM supplemented with 7% calf serum (the murine basal medium, mBM). Two days later, cultures received mBM added with different concentrations of TSE or TCE and cell proliferation was monitored along several days by direct cell counting with a hemacytometer. Cell cultures were maintained as described previously by Alonso-Castro and Salazar-Olivo (2008).

2.5. Effect of TSE and TCE on 2-NBDG uptake in murine and human adipocytes

Murine 3T3-F442A or human adipocytes, differentiated on 96-well fluorescence plates, were incubated for 60 min with PBS containing BSA 1 mg/ml and 80 μM of the fluorescent glucose analog 2-NBDG (Alonso-Castro and Salazar-Olivo, 2008) in the presence of non-toxic concentrations of TSE or TCE. Control cultures were treated with insulin 100 nM or RGZ 10 μM. TSE and TCE effects on 2-NBDG incorporation were also evaluated on 3T3-F442A and human adipocytes pre-incubated with TNF-α 10 ng/ml for 7 days to induce insulin-resistance (Hotamisligil et al., 1994) After incubation, free 2-NBDG was washed out from cultures and fluorescence retained in cell monolayers was measured with a Tecan-GENios fluorescence microplate reader (Tecan, Salzburg, Austria). Values of 2-NBDG incorporation in the absence of insulin were subtracted from those obtained with insulin 100 nM to establish 100% of specific 2-NBDG uptake.

2.6. Effects of TSE and TCE on murine and human adipogenesis

Preconfluent murine 3T3-F442A preadipocytes were treated with murine adipogenic medium (mAM; DMEM containing 10% FBS, 5 μg/ml insulin and 1 μM d-biotin) added with non-toxic concentrations of TSE or TCE. Parallel cultures were fed with non-adipogenic medium (NAM; DMEM containing 4% adult cat serum, 5 μg/ml insulin, and 1 μM d-biotin) (Kuri-Harcuch and Green, 1978) as a negative control. Confluent human preadipocytes were induced to adipogenesis with human adipogenic medium (hAM; L15 containing 5% FBS, insulin 5 μg/ml, RGZ 1 μM, dexamethasone 100 nM, tri-iodothyronine 0.2 nM, and 3-isobutyl-1-methylxanthine 25 μM) (Herrera-Herrera et al., 2009) in the presence of non-toxic concentrations of TSE or TCE. Parallel cultures received human basal medium (hBM; L15 added with 5% FBS) as a negative differentiation control. After seven (murine cells) or thirty days (human cells), adipogenesis was estimated

quantifying intracellular lipid accumulation with oil red O staining (Ramirez-Zacarias et al., 1992). Briefly, oil red O retained in fixed cell monolayers was extracted with 100% isopropyl alcohol and measured spectrophotometrically at 510 nm. For both cell types, the 100% of lipid accumulation was determined subtracting values obtained under non-adipogenic conditions to those achieved in adipogenic conditions.

2.7. Statistical analysis

Experimental values are expressed as mean ± standard deviations of at least three experiments in triplicate. Data were analyzed by using one-way ANOVA. The level of $P \leq 0.05$ was used as criterion of statistical significance. All calculations were done using the JMP 5.1 program (SAS Institute Inc., Cary, NC, USA).

3. Results

3.1. Plant extraction and phytochemical analyses

The lyophilization of TSE and TCE produced 7.69 g and 0.96 g, respectively. Thus, the ratios of the herbal substance to the native herbal drug preparation (DER native) were 6.5:1 for TSE and 52:1 for TCE. The chemical composition of these extracts was characterized by analyzing their content of phenols, flavonoids, and alkaloids, the main compounds reputed as to be responsible for the hypoglycemic properties of many antidiabetic plants (Jung et al., 2006). The chemical characterization of TSE resulted in 12 ± 2.1 GAE g/kg of phenolic compounds, 1.2 ± 0.1 QE g/kg of flavonoids and 20.5 ± 3.4 AE g/kg of alkaloids, whereas the chemical composition of TCE was 6.1 ± 0.9 GAE g/kg of phenolic compounds, 0.8 ± 0.1 QE g/kg of flavonoids, and 0.1 ± 0.04 AE g/kg of alkaloids.

3.2. Determination of non-toxic concentrations of TSE and TCE for in vitro assays

Non-toxic concentrations of TSE and TCE assayable on adipose cells were established by analyzing diverse TSE and TCE concentrations on the cell growth of 3T3-F442A preadipocytes. Added to mBM, only the highest TSE and TCE concentrations tested, 100 μg/ml, decreased the final cell density by 32% and 30%, respectively, as compared to mBM control, whereas lower TSE and TCE concentrations induced only minimal non-significant cell growth reductions (Fig. 1). Thus, the effects of TSE and TCE on 2-NBDG uptake and adipose differentiation were evaluated at concentrations ranging from 1 to 70 μg/ml.

3.3. TSE and TCE stimulate 2-NBDG uptake by insulin-sensitive and insulin-resistant 3T3-F442A adipocytes

To establish whether TSE or TCE stimulate the glucose incorporation into adipocytes, we evaluated their effects on the 2-NBDG uptake in mature 3T3-F442A and human subcutaneous adipocytes. TSE and TCE stimulated 2-NBDG uptake in a concentration-dependent manner in both cell types. In 3T3-F442A adipocytes, TSE stimulated the 2-NBDG uptake by 65%, 116%, and 193% assayed at 1, 30, and 70 μg/ml respectively, compared to the insulin control (Fig. 2A). In these cells, TCE at the same concentrations stimulated 2-NBDG uptake by 37%, 70%, and 112% (Fig. 2B). In human adipocytes, TSE stimulated 2-NBDG incorporation by 56% when applied at 1 μg/ml, by 107% assayed at 30 μg/ml, and by 115% when tested at 70 μg/ml (Fig. 2A), whereas TCE induced 2-NBDG incorporation by 11%, 30% and 54% assayed at the same concentrations as TSE (Fig. 2B).

To further evaluate the antidiabetic potential of TSE and TCE, we assayed their effects on the 2-NBDG uptake in mature murine and human adipocytes made resistant to insulin by TNF-α treatment (see Section 2). In murine diabetic-like adipocytes, TSE stimulated 2-NBDG uptake by 31%, 55%, and 94% when assayed at 30, 50, and 70 μg/ml, respectively (Fig. 2C) whereas TCE induced 2-NBDG uptake by 18%, 35%, and 69% assayed at the same concentrations as TSE (Fig. 2D). In human insulin-resistant adipocytes, TSE 1 μg/ml increased 2-NBDG incorporation by 15%, whereas TSE at 30 and 70 μg/ml induced the glucose uptake by 32% and 70%, respectively (Fig. 2C). In human insulin-resistant cells TCE 1 μg/ml augmented 2-NBDG incorporation by 3% whereas TCE at 30 and 70 μg/ml enhanced the 2-NBDG uptake by 23% and 31%, respectively (Fig. 2D).

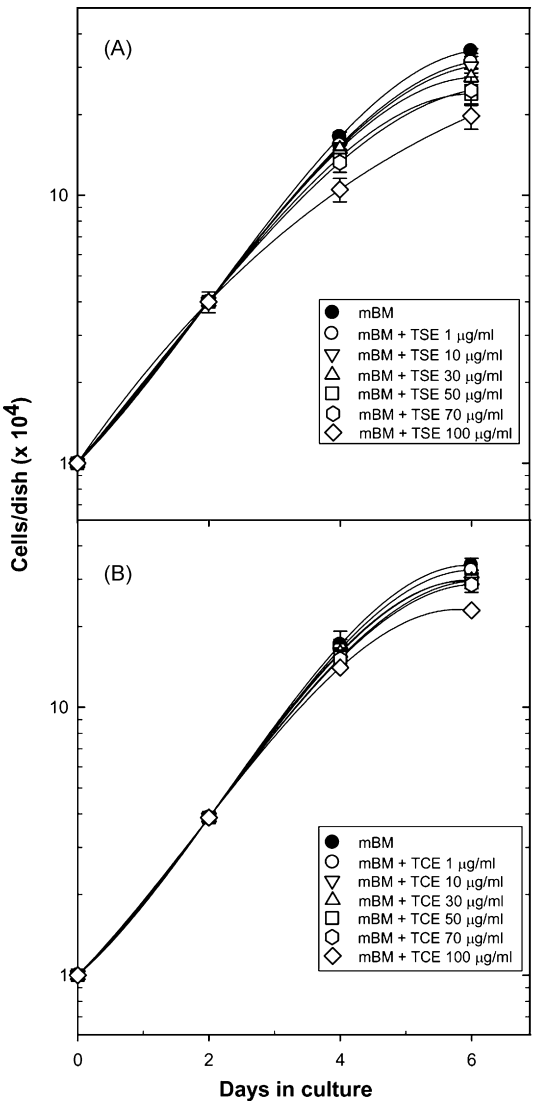

Fig. 1. Effect of aqueous extracts of *Tecoma stans* (TSE) and *Teucrium cubense* (TCE) on cell growth. 3T3-F442A preadipocytes were inoculated in 24-well culture plates (1×10^4 cells/well) in murine basal medium (mBM; see Section 2). After 2 days, cultures were fed with mBM added with different TSE (A) or TCE (B) concentrations. At the indicated days, cell number was determined by direct cell counting in a hemacytometer. The results are presented as the mean ± SD of three independent experiments in triplicate.

3.4. TSE and TCE only minimally affect murine and human adipogenesis

Since the induction of adipogenesis is a side effect of some antidiabetic drugs (Cheng and Fantus, 2005), we also

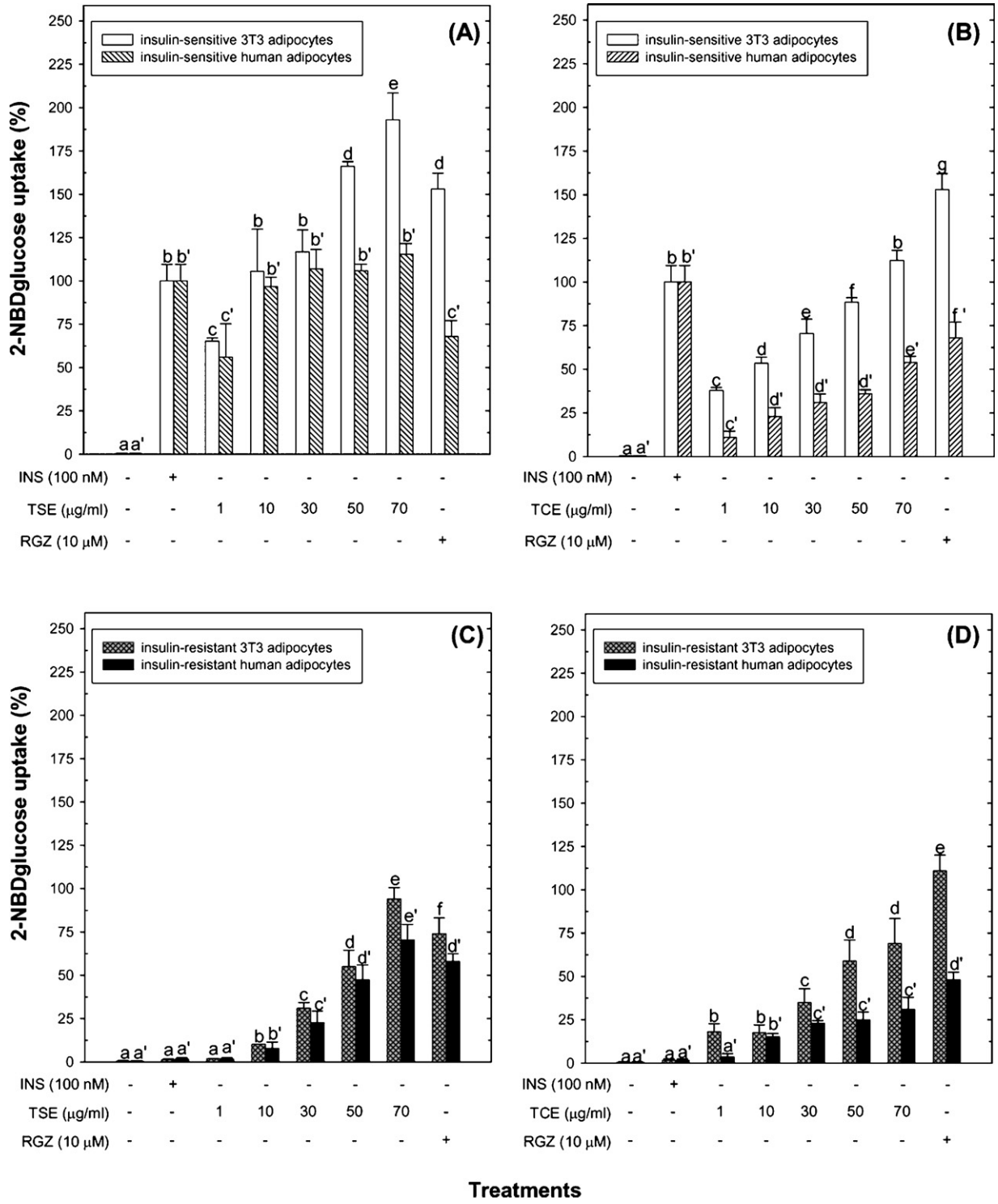

Fig. 2. Effect of TSE and TCE on 2-NBDG uptake in normal and diabetic-like murine and human adipocytes. Insulin-sensitive (A and B) and insulin-resistant (C and D) (see Section 2), 3T3-F442A and human adipocytes were incubated for 60 min with PBS/BSA containing 80 μM of 2-NBDG and the indicated concentrations of TSE (A and C) or TCE (B and D). Control treatments were incubated with insulin 100 nM (INS) or Rosiglitazone 10 μM (RGZ). After incubation, free 2-NBDG was cleared from cultures and fluorescence associated to cell monolayers was measured in a fluorescence reader. The results are presented as the mean ± SD of three independent experiments in triplicate. Lowercase letters indicate significant differences according to ANOVA test ($P \leq 0.05$).

analyzed whether TSE or TCE could affect the development of adipose tissue evaluating their effects on in vitro murine and human adipogenesis. TSE 70 μg/ml induced only a modest increase in 3T3 adipogenesis (117%) respect to the mAM control but did not affect human adipogenesis at any of the tested concentrations (Fig. 3A). TCE concentrations between 1 and 10 μg/ml did not affect 3T3 or human adipogenesis, whereas TCE at 30 μg/ml and 70 μg/ml increased triglyceride content by 137% and 167% in murine preadipocytes, respectively, compared to the mAM control (Fig. 3B). In human preadipocytes,

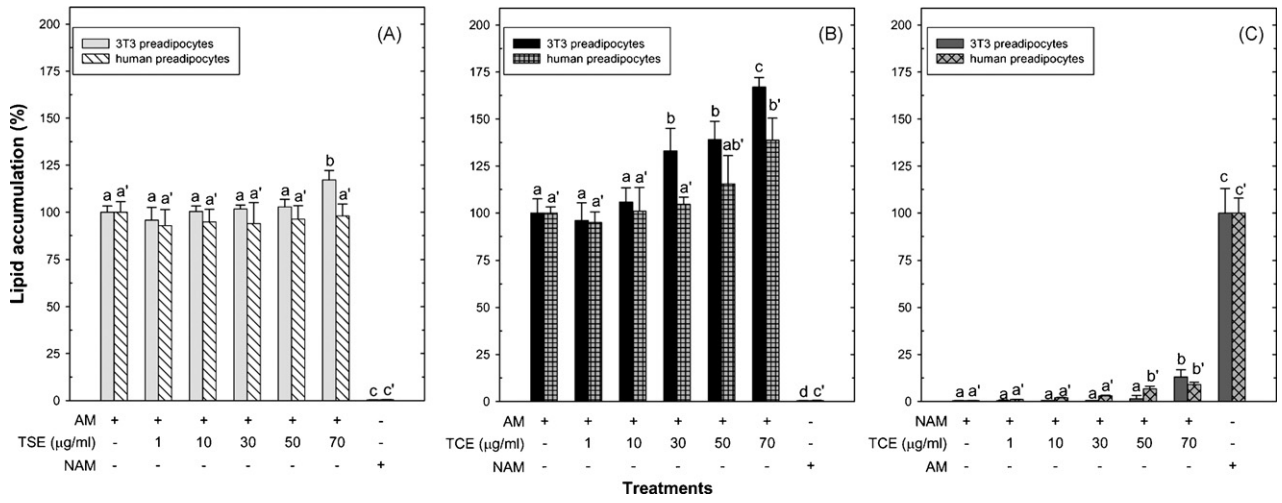

Fig. 3. Effect of TSE and TCE on 3T3 and human adipogenesis. Adipose differentiation of 3T3-F442A and human preadipocytes was induced with whole murine (mAM) or human (hAM) adipogenic medium (see Section 2), respectively, added with non-toxic concentrations of TSE (A) or TCE (B). Control cultures were maintained in non-adipogenic medium (NAM) or human basal medium (hBM). In parallel experiments non-toxic concentrations of TCE (C) were added to NAM or hBM. After 7 days of treatment cultures were fixed and stained with oil red O to estimate triglyceride accumulation. The results are presented as the mean ± SD of three independent experiments in triplicate. Lowercase letters indicate significant differences according to ANOVA test ($P \leq 0.05$). In the figure, AM and NAM denotes adipogenic and non-adipogenic conditions, respectively, as described above for each adipose cell line.

only TCE at 70 μg/ml increased adipogenesis significantly (138%) (Fig. 3B).

The stimulation of murine and human adipogenesis by TCE added to adipogenic medium could be the result of the presence in TCE of (i) adipogenic compounds, or (ii) compounds solely enhancing the effect of serum adipogenic factors added with the culture medium. To test these two possibilities, the effect of TCE on adipogenesis was also evaluated under non-adipogenic culture conditions, a useful strategy for evaluating the adipogenic potential of new compounds (Kuri-Harcuch and Green, 1978). In such culture conditions, TCE only marginally stimulated murine and human adipogenesis by 13% and 9%, respectively (Fig. 3C).

4. Discussion

Tecoma stans and *Teucrium cubense* are extensively used for the empirical treatment of T2-D, but their active principles and hypoglycemic mechanisms are poorly known. Although early studies suggested alkaloids were the main active compounds in *Tecoma stans* (Hammouda et al., 1964; Hammouda and Amer, 1966), their role in the antidiabetic properties in this plant remains controversial (Costantino et al., 2003).

An initial chemical characterization of TSE and TCE showed that these preparations contained higher levels of alkaloids, flavonoids and phenolics than contents reported for other antidiabetic plants. Phytochemical studies showed 0.8 g/kg of polyphenols and 0.05 g/kg of flavonoids in *Aloe ferox*, a plant commonly used in South Africa for the traditional treatment of T2-D (Loots et al., 2007), and 2.5 g/kg of alkaloids in *Equisetum arvense*, used as antidiabetic in Middle Eastern traditional medicine (Shamsa et al., 2008). Thus, TSE contained 15-fold more phenolics and 24-fold more flavonoids that *Aloe ferox* and 8-fold more alkaloids than *Equisetum arvense* whereas TCE contained 7-fold more phenolics and 16-fold more flavonoids that *Aloe ferox*, but 25-fold less alkaloids than *Equisetum arvense*. Therefore, TSE and TCE have high amounts of compounds that could account for the antidiabetic effects of *Tecoma stans* and *Teucrium cubense*, and ongoing experiments in our laboratory will seek to clarify the chemical nature of the hypoglycemic principles in these two extracts.

To gain insight into the mechanisms mediating the antidiabetic properties *Tecoma stans* and *Teucrium cubense*, the effects of non-toxic concentrations of TSE and TCE on glucose uptake in murine and human adipocytes were evaluated. TCE stimulated the 2-NBDG uptake in insulin-sensitive 3T3-F442A and human adipocytes, in a concentration-dependent manner and with potency higher than insulin or Rosiglitazone, whereas TSE showed a stimulatory capacity similar to insulin and slightly lower than thiazolidinedione. TSE and TCE also stimulated the 2-NBDG uptake in insulin-resistant adipocytes. In these diabetic-like cells, insulin did not stimulate 2-NBDG uptake whereas TSE and TCE stimulated uptake in a pattern similar to that exerted on insulin-sensitive adipocytes, although with lower potency. These results suggest that stimulation of glucose uptake in insulin-targeted tissues is one of the mechanisms by which *Tecoma stans* and *Teucrium cubense* exert their antidiabetic effects. The possibility that antidiabetic properties of *Tecoma stans* could be due to insulin-secreting effect was previously excluded (Lozoya-Meckes and Mellado-Campos, 1985) and recently an alpha-glycosidase inhibitory activity was reported in aqueous extracts of this plant (Aguilar-Santamaría et al., 2009). Thus, our results show a new biological effect for *Tecoma stans* and *Teucrium cubense* and suggest a new antidiabetic mechanism of these plants, highly promising for the development of new T2-D therapies. Our results also suggest that TSE and TCE stimulate glucose uptake by activating the insulin signaling pathway and that both extracts partially reverse the insulin resistance induced in fat cells by TNF-α. To clarify the molecular mechanisms of action of TCE and TSE, as well as their active compounds, experiments currently underway in our laboratory address the effect of these preparations on the insulin signaling pathway in adipose cells.

The stimulation of glucose uptake by fat cells has been documented for extracts of other antidiabetic plants such as *Toona sinensis* (Yang et al., 2003) and *Paeonia suffruticosa* (Lau et al., 2007), but the effect of these extracts on insulin-resistant adipocytes was not evaluated. Recently we showed that aqueous extracts from *Cecropia obtusifolia* and *Guazuma ulmifolia* also stimulated glucose uptake in both insulin-sensitive and insulin resistant 3T3-F442A adipocytes (Alonso-Castro and Salazar-Olivo, 2008; Alonso-Castro et al., 2008), although their effects were less potent that those of TSE and TCE. Thus, the results presented here suggest that *Tecoma stans* and *Teucrium cubense* could be more suitable candidates for the search of new hypoglycemic compounds.

Results present here also indicate that TSE does not increase 3T3 or human adipogenesis induced by adipogenic medium, a negative side effect reported for oral hypoglycemics (Cheng and Fantus, 2005). Although TCE significantly increased adipogenesis induced in both cell types by adipogenic culture conditions, this preparation did not exert adipogenic effects assayed in the absence of serum adipogenic signals (non-adipogenic medium). These results demonstrate that TCE lacks compounds intrinsically adipogenic for murine and human preadipocytes and suggest that this extract does not induce murine or human obesity. The absence of effects on the adipose development in TCE and TSE, together with their proven effects on glucose uptake, increases the value of these extracts as a possible source of new antidiabetic compounds.

In summary, this work shows that *Tecoma stans* and *Teucrium cubense* contain water-soluble compounds stimulating glucose uptake in murine and human adipocytes, both sensitive and resistant to insulin, without significantly affecting their adipogenesis. Our results provide a more detailed view of the antidiabetic potential of these two plant species and suggest for the first time a mechanism of action for the antidiabetic properties of *Teucrium cubense* and document a new antidiabetic mechanism for *Tecoma stans*. Future studies will address the molecular mechanisms by which these plants and their active compounds regulate glucose uptake by adipose and muscle tissues.

Acknowledgements

AJAC (210841), RZB (211445) and PCL (270638) were endowed with graduate fellowships from CONACYT. AJAC also received a special support from IPICYT (SA-157/2008). We thank Dr. Robert W. Jones for critically reading the manuscript.

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