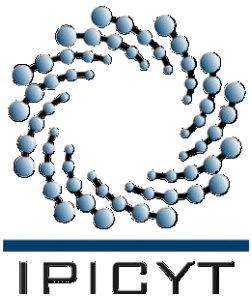

INSTITUTO POTOSINO DE INVESTIGACIÓN CIENTÍFICA Y TECNOLÓGICA, A.C.

POSGRADO EN CIENCIAS EN BIOLOGÍA MOLECULAR

SEARCH FOR BIOACTIVE COMPOUNDS IN AMERICAN MISTLETOES AND THEIR MECHANISMS OF ACTION

Tesis que presenta

Mónica Gabriela Sánchez Salazar

Para obtener el grado de

Maestra en Ciencias en Biología Molecular

Codirectores de la Tesis:

Dr. Luis Antonio Salazar Olivo

Dr. Francisco Elihú Bautista Redonda

San Luis Potosí, S.L.P., Agosto de 2017

IPICYT

Constancia de aprobación de la tesis

La tesis ***"Search for bioactive compounds in American mistletoes and their mechanisms of action"*** presentada para obtener el Grado de Maestra en Ciencias en Biología Molecular fue elaborada por Mónica Gabriela Sánchez Salazar y aprobada el **veintitrés de agosto de dos mil diecisiete** por los suscritos, designados por el Colegio de Profesores de la División de Biología Molecular del Instituto Potosino de Investigación Científica y Tecnológica, A.C.

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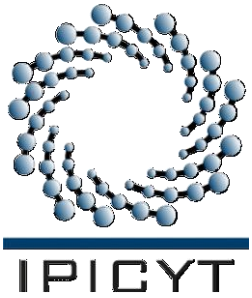

Créditos Institucionales

Esta tesis fue elaborada en el Laboratorio de Biotecnología Médica y Pecuaria de la División de Biología Molecular del Instituto Potosino de Investigación Científica y Tecnológica, A. C. y en el Laboratorio de Productos Naturales del Centro de Investigación, Innovación y Desarrollo para las Zonas Áridas, bajo la codirección de los doctores Luis Antonio Salazar Olivo y Francisco Elihú Bautista Redonda. El manuscrito se redactó siguiendo las normas para autor de la revista Journal of Ethnopharmacology.

Durante la realización del trabajo, la autora recibió una beca académica del Consejo Nacional de Ciencia y Tecnología (número de registro 588891).

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MAESTRA EN CIENCIAS EN BIOLOGÍA MOLECULAR

sustentó la C.

Mónica Gabriela Sánchez Salazar

sobre la Tesis intitulada:

Search for bioactive compounds in American mistletoes and their mechanisms of action

que se desarrolló bajo la dirección de

Dr. Francisco Elihu Bautista Redonda
Dr. Luis Antonio Salazar Olivo

El Jurado, después de deliberar, determinó

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Dedicatorias

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RESUMEN

Búsqueda de compuestos bioactivos en muérdagos Americanos y sus mecanismos de acción

Relevancia etnofarmacológica: Las propiedades medicinales atribuidas a los muérdagos Americanos siguen siendo empíricas y aún se desconocen sus compuestos activos y mecanismos de acción.

Objetivo del estudio: Analizar los mecanismos de los efectos vasorrelajantes y anti-hiperglicémicos de preparaciones de *Psittacanthus calyculatus* y *Phoradendron velutinum* y evaluar su potencial anti-adipogénico.

Materiales y métodos: Los efectos de preparaciones de muérdagos en la expresión de la sintasa de óxido nítrico endotelial (*NOS3*) y marcadores adipogénicos relevantes en adipocitos humanos se evaluó por RT-PCR y su potencial anti-diabético se evaluó por incorporación de 2-NBDG en adipocitos y ensayos de inhibición de *α*-glucosidasa y *α*-amilasa.

Resultados: Las fracciones de acetato de etilo (EA) y acuosa (WT) de *P. calyculatus* y el extracto crudo (CE) y fracción WT de *Ph. velutinum* incrementaron al doble la expresión de *NOS3* en adipocitos inducidos o maduros. Todas las preparaciones promovieron 80-125% la incorporación de 2-NBDG en adipocitos maduros. Todas las preparaciones de *P. calyculatus* y las fracciones hexánica (HX) y EA de *Ph. velutinum* inhibieron 70-98% la actividad enzimática de *α*-glucosidasa. Todas las preparaciones redujeron la expresión de al menos dos marcadores adipogénicos en adipocitos inducidos y todas las preparaciones de *Ph. velutinum* y EA de *P. calyculatus* redujeron la expresión de al menos un marcador adipogénico en adipocitos maduros.

Conclusiones: *Psittacanthus calyculatus* y *Phoradendron velutinum* estimulan la expresión de *NOS3* y reducen la de marcadores adipogénicos en adipocitos humanos en cultivo, mientras que promueven la captación de glucosa e inhiben la actividad de la enzima *α*-glucosidasa. Estos efectos pueden explicar las propiedades antihipertensivas y anti-diabéticas atribuidas a estas plantas.

Palabras clave: muérdagos Americanos, sintasa del óxido nítrico, anti-hiperglicémico, adipogénesis.

ABSTRACT

Search for bioactive compounds in American mistletoes and their mechanisms of action

Ethnopharmacological relevance: The putative medicinal properties of American mistletoes remain empirical and their active compounds and mechanisms of action remain unknown.

Aim of the study: To analyze the mechanisms of the vasorelaxant and anti-hyperglycemic effects of *Psittacanthus calyculatus* and *Phoradendron velutinum* preparations and to assess their anti-adipogenic potential.

Materials and Methods: The effects of mistletoe preparations on the expression of endothelial nitric oxide synthase (*NOS3*) and relevant adipogenic markers by cultured human adipocytes were evaluated by RT-PCR and their anti-diabetic potential was assessed by adipocyte 2-NBDG incorporation and by an *α*-glucosidase inhibition assay.

Results: Ethyl acetate (EA) and aqueous (WT) fractions of *P. calyculatus* and crude extract (CE) and WT fraction of *Ph. velutinum* increased by 2-fold the expression of *NOS3* in induced or mature adipocytes. All preparations promoted an 80-125% 2-NBDG incorporation in mature adipocytes. All *P. calyculatus* preparations and hexane (HX) and EA fractions of *Ph. velutinum* inhibited 70-98% *α*-glucosidase enzymatic activity. All preparations reduced the expression of at least two adipogenic markers in induced adipocytes and all *Ph. velutinum* preparations and EA of *P. calyculatus* reduced the expression of at least one adipogenic marker in mature adipocytes.

Conclusions: *Psittacanthus calyculatus and Phoradendron velutinum* stimulate the expression of *NOS3* and decrease that of relevant adipogenic markers in cultured human adipocytes, while promoting glucose uptake by these cells and inhibiting the enzymatic activity of *α*-glucosidase. These effects could explain the antihypertensive and anti-diabetic properties attributed to these plants.

Keywords: American mistletoes, nitric oxide synthase, antihyperglycemic, adipogenesis.

Search for bioactive compounds in American mistletoes and their mechanisms of action

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Abstract

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1. Introduction

Mistletoes are evergreen hemiparasitic plants from mainly the Loranthaceae and Viscaceae families growing on broadleaved and coniferous trees of multiple species (Marvibaigi et al., 2014). Mistletoes have long been used worldwide in traditional medicine (Guimarães, 2006). Recognized therapeutical properties for these plants include cytotoxicity against cancer cells (Alonso-Castro et al., 2013; Jacobo-Salcedo et al., 2011; Li et al., 2002; Sauviat et al., 1985), immuno-modulatory (Alonso-Castro et al., 2012; Fernández et al., 1998), anti-microbial (Jacobo-Salcedo et al., 2011; Rivero-Cruz et al., 2005), anti-hypertensive (Lorenzana-Jiménez et al., 2009; Rios et al., 2012; Rodríguez-Cruz et al., 2003) and anti-hyperglycemic effects (Avila-Acevedo et al., 2012; Careaga-Olivares et al., 2006; Ramírez-Espinosa et al., 2013).

Mistletoes are a great source of bioactive compounds such as toxins (Mellstrand and Samuelsson, 1973), saponins (Gonzalez et al., 2000; Sánchez-Arreola et al., 2004), flavonoids (Dossaji et al., 1983; Varela et al., 2004), triterpenes (Kashiwada et al., 1998; Ramírez-Espinosa et al., 2013; Rios et al., 2001), phytosterols (Marín-Canchala et al., 2013) and non-protein aminoacids (Bah, et al., 2011). Said compounds have been isolated from genres like *Phoradendron, Psittacanthus, Struthanthus, Arceuthobium* and *Ligaria*.

Psittacanthus calyculatus is known as “injerto” or “muérdago verdadero” in central region of Mexico and it is empirically used as a remedy for diabetes and hypertension (Guimaraes, 2006; Hernández et al. 2015). There is evidence of vasorelaxant effects exerted by aqueous and ethanol extracts of *P. calyculatus*

(Cervantes-Badillo, 2006; Rodríguez-Cruz et al., 2003) and antihyperglycemic properties of *P. calyculatus* methanolic extract (Ávila-Acevedo et al., 2012).

Phoradendron velutinum is another American mistletoe, known as “injerto de cazahuate” present also in central region of Mexico, which is used to treat anxiety and depression (Guzmán-Gutiérrez et al., 2014). Up to now, few studies have been carried out on the biochemical composition and bioactivities of components of this mistletoe. Other *Phoradendron* species have proved to exert vasorelaxant (Rios et al., 2012) and hypoglycemic (Careaga-Olivares et al., 2006) effects *in vitro* and *in vivo* assays.

Previous work from our laboratory showed that an aqueous extract of *P. calyculatus* exerts a vasorelaxant effect on guinea pig aortic rings in an endothelium-dependent manner, blocked by the eNOS inhibitor L-NAME (Cervantes-Badillo, 2006). Although, the bioactive compounds and mechanism of action responsible of such effect remain unknown.

To gain insight into the vasorelaxant effect exerted by *P. calyculatus* we evaluated the effect of different preparations on the expression of the endothelial nitric oxide synthase (eNOS) gene, we also evaluated the anti-hyperglycemic and anti-adipogenic effects of *P. calyculatus* and *Ph. velutinum* preparations.
Thus, we decided to evaluate the effect of *P. calyculatus* and *Ph. velutinum* extracts on the expression of *NOS3* gene. As for the anti-hyperglycemic potential, we decided to evaluate the effect of both species extracts and fractions of different polarity on the glucose uptake by human subcutaneous adipocytes *in vitro* and on the inhibition of *α*-glucosidase enzyme. Plus, we evaluated the expression of adipogenic markers to measure the anti-adipogenic potential.

2. Materials and methods

2.1 Plant material

Samples of *Psittacanthus calyculatus* (DC.) G. Don., growing on *Prosopis sp.* were collected in Querétaro, México (February 2016), and samples of *Phoradendron velutinum* (DC.) Eichler growing on *Crataegus sp.,* were collected in Valle de los Fantasmas, Sierra de Álvarez, San Luis Potosi, Mexico (February 2016). Each sample was taxonomically validated by a specialist and a voucher specimen of each sample was deposited in the herbarium of Universidad Autónoma de Querétaro (QMEX).

2.2 Preparation and partitioning of crude extracts

500 g of pulverized dried leaves were trice extracted by maceration in 1000 mL of dichloromethane:methanol (DCM:MeOH) 1:1 for 48 h. The crude extracts (CE) were recovered, filtered and evaporated under reduced pressure. CE was dissolved in hexane (HX) and liquid-liquid extracted three times by a mixture MeOH:H_2O 5:1. HX phase was recovered and evaporated under reduced pressure to obtain a low polarity fraction. Hydroalcoholic phase was evaporated under reduced pressure to eliminate the residual methanol and then trice partitioned with ethyl acetate (EA) to obtain highly polar and medium polar fractions. EA fractions were evaporated to dryness with a rotary evaporator and aqueous fractions (WT) were lyophilized. The processed fractions were stored at room temperature until use on cell cultures.

2.3 Thin layer chromatography for extracts and fractions

CE, HX and EA fractions were developed in silica gel plates using different elution systems: HX/EA 7:3 × 2, 2:3 × 3, 3:7 × 3; DCM/MeOH 95:5 × 2; 85:15 × 2; 80:20 × 2, 70:30 × 2; Acetone/EA 7:3 × 3, and $CHCl_3$/MeOH 1:1. UV light (254 nm) and a solution of $CeSO_4$ dissolved in H_2SO_4 2N followed by heating were used to reveal the plates.

2.4 Analytical and semipreparative HPLC-UV

For analytical conditions, the WT fraction of *Ph. velutinum* was dissolved in MeOH to a concentration of 0.5 mg/mL and submitted to HPLC-UV analysis on an Agilent 1200 series Rapid Resolution Liquid Chromatograph equipped with a binary pump and photodiode array detector. The parameters employed to carry out the analysis were: a column Purospher STAR RP-18 column (Merckmillipore, 4.5 x 250 mm, 5 μm) with an isocratic elution of MeOH/acetic acid in H_2O 0.5% in a proportion of 60:40, a flow of 0.4 mL/min and an injection volume of 20 μL, wavelength detection of 254 nm at room temperature.

For semipreparative conditions, the WT fraction of *Ph. velutinum* was dissolved in MeOH to a concentration of 6.0 mg/mL and submitted to HPLC (Waters chromatograph with 600E multisolvent delivery system and 996 photodiode array detector) on a Symetry C18 column (Waters, 19 x 150 mm, 7 μm) with an isocratic elution of MeOH/H_2O in a proportion of 60:40, a flow of 4.1 mL/min, and an injection volume of 500 μL each time. The peaks with a retention time of 12.0 and 16.4 min were collected.

2.5 Structural elucidation

ESIMS analysis. The WT fraction of *Ph. velutinum* was dissolved in MeOH at a concentration of 1mg/mL and analyzed by direct injection on a Bruker Daltonics Squire 6000 mass spectrometer. The data were acquired in mode of positive detection, and in the m/z window of 100-1000 uma.

NMR analysis. WT fraction of *Ph. velutinum* (5 mg) was dissolved in 0.75 mL of DMSO-d_6 and transferred to an NMR tube. The NMR data were acquired on a Bruker Avance III spectrometer (400 MHz) and processed using MestreLab software.

2.6 Materials for experiments in vitro

Human adipose stromal cells (preadipocytes) were derived from an inguinal subcutaneous adipose tissue biopsy from a male patient (20 months old) subjected to corrective surgery in the Children and Women Hospital (San Luis Potosí, Mexico). An informed consent from the parents according to the hospital Ethics Committee was obtained. For primary preadipocyte isolation, tissue was disaggregated as described (Herrera-Herrera et al., 2009). Briefly, the vascular stromal fraction was obtained from washing and finely chopping the sample, digesting with collagenase type II, centrifuging at low speed and resuspending the cells.

Leibovitz's L15 medium was obtained from GIBCO, fetal bovine serum (FBS) and calf serum (CS) were obtained from Hyclone. Insulin, biotin, calcium pantothenate, cortisol, transferrin, triiodothyronine (T3), dexamethasone (DEX), 3-isobutyl-1-methylxanthine (IBMX), penicillin, streptomycin, collagenase type II and

dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich. Rosiglitazone (RGZ) was purchased from Cayman Chemical. 3-(4, 5-dimethylthiazol-2-yl)–2,5-diphenyltetrazoliumbromide (MTT) and sodium dodecyl sulphate (SDS) were purchased from Roche, and 2-Deoxy-2-[(7-nitro-2,1,3-benzoxadiazol-4-yl)amino] (2-NBDG) was purchased from Invitrogen.

2.7 Cell viability assay

Human preadipocytes were seeded at 5×10^3 cells/well in 96 well plates and incubated for 24 h. The cells were treated with crude extracts and fractions at concentrations 0.01, 0.1, 1, 10 and 50 µg/mL for 48 h. DMSO in a 0.05% v/v concentration was used as vehicle control. Next, fresh medium (100 µL) together with MTT (0.5 mg/mL) was added to each well and the plate was incubated at 37°C for 4 h. Afterwards, SDS (100 µL) was added to dissolve the formazan complex overnight. Absorbance was detected at 540 nm by using a microplate reader.

2.8 Differentiation of human preadipocytes

Human preadipocytes were grown in Leibovitz's L15 medium (L15) containing 5% fetal bovine serum, 5% calf serum and antibiotics (80 U/mL penicillin and 80 µg/mL streptomycin). Two days after confluence, the cells were stimulated for differentiation with differentiation medium (L15 containing 5% FBS, 100 nM insulin, 33 µM biotin, 17 µM calcium pantothenate, 100 nM cortisol, 5 µg/mL transferrin, 2 nM T3, 100 nM DEX, 500 µM IBMX, 1 µM RGZ and antibiotics) for 6 days and subsequently cultured in maintenance medium (L15 containing 5% FBS, 100 nM insulin, 33 µM biotin, 17 µM calcium pantothenate, 100 nM cortisol, 5

µg/mL transferrin, 2 nM T3 and antibiotics) for 10 more days. Two concentrations of *P. calyculatus* and *Ph. velutinum* preparations (10 and 30 µg/mL) were added along with the differentiation medium for subsequent RNA extraction.

2.9 Expression of NOS3 and adipogenic molecular markers

RNA was extracted from cultured young and mature human adipocytes with Trizol according to the manufacturer's instructions. Subsequently, 1 µg total RNA was reverse transcribed into cDNA using the manufacturer's procedure. The molecular markers analyzed were fatty acid binding protein 4 (*FABP4*), Krüppel-like factor 4 (*KLF4*), peroxisome proliferator-activated receptor gamma (*PPARG*), CCAT/enhancer binding protein alpha (*CEBPA*) and leptin (LEP). Endothelial nitric oxide synthase (*NOS3*) primers were used to confirm the expression of this gene in adipocytes and to later evaluate the effect of crude extract and fractions on *NOS3* expression. The primers used are noted in Table 2.

2.10 Determination of glucose uptake with 2-NBDG

Glucose uptake in cultured human adipocytes was measured using the fluorescent glucose analog 2-NBDG. Mature adipocytes were starved with phosphate buffered saline (PBS) 0.1% albumin for 1 h. Subsequently, 80 µM 2-NBDG was added to the PBS 0.1% albumin solution for 1 h containing different concentrations of *P. calyculatus* and *Ph. velutinum* preparations (10 and 30 µg/mL doses were chosen based on preliminary MTT assay), 100 nM insulin or 10 µM RGZ. The 2-NBDG fluorescence intensity was measured with a microplate reader at an excitation wavelength of 465 nm and an emission wavelength of 540 nm.

2.11 α-Glucosidase inhibitory activity

Inhibition of the enzyme was evaluated by the PNPG method (p-nitrophenyl-*α*-D-glucopyranoside) with some modifications. In a 96-well plate, reaction mixture containing 25 *μ*L sodium phosphate buffer (20 mM, pH = 6.9) or 25 *μ*L of varying concentrations of *P. calyculatus* and *Ph. velutinum* preparations (10, 25, 50, 250, 500, 1000 *μ*g/mL) dissolved in sodium phosphate buffer and 25 *μ*L α-glucosidase (0.2 U/mL), was preincubated at 20°C for 10 min. Then, 50 *μ*L of PNPG (2 mM) was added as a substrate and incubated further at 20°C for 20 min. The reaction was stopped by adding 50 *μ*l Na_2CO_3 (0.2 M). The absorbance of the released p-nitrophenol was measured at 405 nm using a microplate reader. Acarbose at various concentrations (0.1–1.5 mg/mL) was included as a standard. Reaction without test substance was set up in parallel as a control and each experiment was performed in triplicates. The results were expressed as percentage inhibition, which was calculated using the formula,

$$\text{Inhibitory activity (\%)} = (1 - As/Ac) \times 100$$

Where, As is the absorbance in the presence of test substance and Ac is the absorbance of control.

3. Results

3.1 Yield and chemical composition of Psittacanthus calyculatus and Phoradendron velutinum

We obtained crude extracts from the leaves of *P. calyculatus* and *Ph. velutinum* to explore their beneficial effects in diseases like hypertension, diabetes and obesity. We obtained 247.78 g and 172.73 g of crude extract with yields of 16% and 35%, respectively. The yields for each fraction are shown in Table 1.

CE, HX and EA were analyzed by TLC. Both species have triterpenes, which reveal as pink, and flavonoid compounds, which reveal as yellow (data not shown). *P. calyculatus* CE was rich in low polarity and high polarity compounds, with the HX fraction being the most abundant, whilst *Ph. velutinum* CE was rich in medium and high polarity compounds, since the EA and WT fractions were the most abundant.

WT fraction from *Ph. velutinum* was further analyzed because it resembled a pure chemical compound. Subsequent chromatographic, $ESIMS^+$ and NMR analyses revealed that the fraction was a mixture of two major components (Figures 1-3).

The HPLC-UV analysis showed to main peak with retention times of 8.6 and 10.1 min. The portion observed for both in the mixture was 2:1 (Figure 1). In the same way, the ^1H NMR spectrum (Figure 3) displayed a mixture in relation 2:1, considering the relative integrals of the signals at δ_H 7.93 (d, J = 8.8 Hz) and 7.33 (d, J = 8.6 Hz). These signals are distinctive of hydrogens in aromatic rings and possess similar constant coupling, but the difference in the integral value for each

signal indicated the presence of a mixture. Comparison of the ^{1}H NMR data of the WT fraction with those described in the literature, allowed to infer that the mixture is composed by two flavonoid glycosides. In addition, the ESIMS^{+} spectrum (Figure 2) showed two peaks with *m/z* 485 $[M + H]^{+}$ and 469 $[M + H]^{+}$, which suggested that the main compounds differ by an oxygen atom. In order to obtain more information about their structures, a MS/MS analysis was carried out. In this analysis, the molecular ions (*m/z* 485 and 469) were isolated in an individual way. These selected ions produced fragment peaks at *m/z* 323 $[M -162 + H]^{+}$ (Figure 4) and *m/z* 307 $[M – 162 + H]^{+}$ (Figure 5). The loss of the same mass fragment indicated a close similarity in their structures and confirmed the presence of a glucose unit in each compound.

3.2 Effect of P. calyculatus and Ph. velutinum on cell viability in human preadipocytes.

Human preadipocytes were exposed to various concentrations (0.01 – 50 µg/mL) of *P. calyculatus* and *Ph. velutinum* extracts and fractions. The effect of the preparations was determined using the MTT cytotoxicity assay (Figure 6). The result of the MTT assay indicated that 48 h exposure of cells to *P. calyculatus* and *Ph. velutinum* preparations exerted no negative effect on the cell viability at concentration up to 50 µg/mL, in exception of the HX and WT fractions from *P. calyculatus* and to a lesser extent, EA and WT fractions from *Ph. velutinum*.

3.3 *P. calyculatus and Ph. velutinum up-regulate NOS3 expression in human subcutaneous adipocytes*

To learn if we could evaluate the potential anti-hypertensive effect of *P. calyculatus* and *Ph. velutinum* in human subcutaneous adipocytes, we used RT-PCR with specific primers (Table 2) to assess the expression of endothelial nitric oxide synthase (*NOS3*) mRNA in proliferative preadipocytes, quiescent preadipocytes, induced adipocytes and terminal adipocytes. The results show that *NOS3* expression is increased further into the differentiation process, which makes our strain hASCB1 a suitable model to evaluate the effect of *P. calyculatus* and *Ph. velutinum* preparations on *NOS3* expression.

Previous work from our lab found out that an aqueous extract from *P. calyculatus* exerts a vasorelaxant effect on rat aortic rings in an endothelium-dependent manner and such effect is related to nitric oxide production. Thus, we analyzed by RT-PCR the expression of *NOS3* in induced adipocytes and terminal adipocytes in presence of *P. calyculatus* and *Ph. velutinum* preparations. The analysis shows that EA and WT fractions from *P. calyculatus* increased *NOS3* expression in induced adipocytes (Figure 7A), which matches with our previous results, but in the differentiated state, HX and WT are the fractions which show greater effect (Figure 7B). In the case of *Ph. velutinum*, all fractions appear to shut down the expression of *NOS3* in induced adipocytes (Figure 7C), but in terminal adipocytes, CE and WT show an up-regulation of the gene's expression (Figure 7D). These results confirm the ethnopharmacological use of infusions from these plants in the treatment of hypertension.

3.4 P. calyculatus and Ph. velutinum down-regulate adipogenic genes expression

Since mistletoes are used by folk medicine to treat a great variety of health conditions, we decided to also evaluate their potential anti-adipogenic effect by assessing the expression of adipogenic marker genes *FABP4*, *KLF4*, *PPARG*, *CEPBA* and *LEP*.

CE from *P. calyculatus* notably reduced the expression of *PPARG, CEBPA, FABP4* and *LEP* expression in a dose dependent manner in freshly induced adipocytes. HX fraction notably reduced the expression of *PPARG* and *LEP* in a dose-dependent manner, but had mild effect on *CEBPA* and no effect on *FABP4* expression. EA fraction only had a visible effect in a concentration of 30 µg/mL on the expression of *PPARG* and *FABP4*, and a mild effect on *CEBPA* and *LEP*. WT fraction notably reduced the expression of *LEP* in a similar way as CE, and had a mild effect on the expression of *PPARG*, *FABP4* and *CEBPA* in a concentration of 30 µg/mL (Figure 8).

In terminal adipocytes, CE from *P. calyculatus* down-regulated the expression of *PPARG, CEBPA* and *LEP* and it had no effect on *FABP4* expression and it increased the. On the contrary, CE increased the expression of *KLF4* in a dose-dependent manner. HX fraction increased the expression of *FABP4* and *KLF4*, it had no effect on *PPARG* and *CEBPA* expression and it mildly reduced the expression of *LEP*. EA fraction mildly increased the expression of KLF4 in a dose-dependent manner, it mildly reduced the expression of *PPARG* and *LEP* but had a better effect on the expression of *CEBPA* and showed no effect on *FABP4*. WT fraction showed no inhibitory effect on the expression of markers *PPARG*, *CEBPA* and *LEP*, but it increased the expression of *FABP4* and *KLF4* in a dose-dependent manner (Figure 9).

On the other hand CE from *Ph. velutinum* reduced the expression of *PPARG, CEBPA* and *FABP4* on a dose-dependent manner in freshly induced adipocytes. CE also reduced the expression of *LEP*, but not in a dose-dependent manner. HX fraction showed a down-regulation of all adipogenic markers in a dose-dependent manner. EA fraction reduced *CEBPA* expression in a similar way as HX and EA had a stronger inhibitory effect on the expression of *PPARG*, *LEP* and *FABP4* in a dose dependent-manner. WT fraction had no effect on the expression of *PPARG*, but showed the greatest inhibitory effect on the expression of *CEBPA*. In a lesser extent, this fraction also down-regulated the expression of *LEP* and *FABP4* (Figure 10).

In terminal adipocytes, CE from *Ph. velutinum* reduced the expression of *PPARG*, *CEBPA* and *FABP4* in a dose-dependent manner, but had no effect on *LEP* and *KLF4* expression. HX fraction reduced the expression of *PPARG*, *CEBPA* and *FABP4* but not in a dose-dependent manner; on the other hand, this fraction up-regulated the expression of *LEP* and *KLF4* in a dose-dependent manner. EA fraction had no effect on *KLF4* and *LEP* expression, but it down-regulated the expression of *PPARG*, *FABP4* and *CEBPA* in a dose-dependent manner. Finally, WT fraction clearly down-regulated the expression of *PPARG*, *CEBPA* and *FABP4* and had a mild effect on *LEP* expression in a dose-dependent manner, but this fraction also had no effect on *KLF4* expression (Figure 11).

3.5 P. calyculatus and Ph. velutinum promote 2-NBDG uptake in human adipocytes

P. calyculatus and other mistletoe species are also used in folk medicine to treat diabetes. To establish the regulatory effect of *P. calyculatus* and *Ph. velutinum* on glucose consumption in peripheral tissue, we performed a glucose uptake assay using 2-NBDG in human adipocytes (Figure 12). The results indicated that *P. calyculatus* and *Ph. velutinum* CEs and fractions stimulated 2-NBDG uptake in a similar way than that of insulin, but not in a dose-dependent manner.

3.6 *P. calyculatus and Ph. velutinum inhibit α-glucosidase enzymatic activity*

Another possible mechanism for the anti-diabetic effect observed in folk medicine for these plants is the inhibition of glycolytic enzymes. Herein, we evaluated *P. calyculatus* and *Ph. velutinum* preparations for their inhibitory effect on the *α*-glucosidase enzyme by an *in vitro* method.

P. calyculatus CE and HX, EA and WT fractions (at a concentration of 0.5 mg/mL) exhibited 95, 89, 97 and 98% of *α*-glucosidase inhibitory activity (Figure 13A), with the IC_{50} values being 44, 44, 71 and 49 *μ*g/mL, respectively. Whereas *Ph. velutinum* fractions HX and EA (at a concentration of 1 mg/mL) exhibited 91 and 62% α-glucosidase inhibitory activity (Figure 13B) with and IC_{50} value of 60 *μ*g/mL for the HX fraction; CE and WT of *Ph. velutinum* showed no inhibitory activity (data not shown). Acarbose was used as a standard reference drug, which showed *α*-glucosidase inhibitory activity with an IC_{50} of 700 *μ*g/mL. Among all, *P. calyculatus* fractions EA and WT showed best enzyme inhibitory activity.

4. Discussion

Mistletoes have been employed in folk medicine to treat illnesses like hypertension and diabetes. Some reports show antihypertensive activity of some mistletoe preparations, for example a crude ethanol extract of *P. calyculatus* induced the synthesis or release of nitric oxide, since it relaxed rat aortic rings in an endothelium-dependent manner, and this effect was reverted by the nitric oxide synthase inhibitor L-NAME (Rodríguez-Cruz et al., 2003). Another study also showed that *P. calyculatus* aqueous extract induced endothelium-dependent vasodilation (Ibarra-Alvarado et al., 2010).

Psittacanthus calyculatus is a hemiparasitic plant which grows in mesquite trees (*Proposis laevigata*) and is used in Mexico as a remedy for diabetes and hypertension (Hernández et al. 2015). *Phoradendron velutinum* is used to treat anxiety and tension (Guzmán-Gutiérrez et al., 2014). Currently, there are no studies on the biological effects by *Phoradendron velutinum*, but other *Phoradendron* species have shown anti-hypertensive and anti-diabetic properties, like *Phoradendron tomentosum* (Careaga-Olivares et al., 2006), *Phoradendron reichenbachianum* (Ramírez-Espinosa et al., 2013), *Phoradendron brachystachyum* (López-Martínez et al., 2013) and *Phoradendron serotinum* (Alonso-Castro et al., 2012).

In this work we studied the potential anti-hypertensive, anti-diabetic and anti-adipogenic effects of crude extracts and fractions obtained of the mistletoe species *Psittacanthus calyculatus* and *Phoradendron velutinum*, two native plant species employed in folk medicine that can be a good source of bioactive compounds. We obtained organic extracts and fractions of increasing polarity from leaves of both

plants, which allowed us to evaluate the presence of molecules with vasorelaxant, hypo-glycemic and anti-adipogenic activity of different natural chemistry.

Cardiovascular disease, hyperglycemia and obesity are closely related because insulin resistance increases plasma levels of free fatty acids and glucose. Then, hyperglycemia and free fatty acids increase reactive oxygen species and decrease nitric oxide, a key molecule for vasculature relaxation (Storniolo et al., 2014). Thus, it is important to find novel natural compounds that have both antihyperglycemic and vasorelaxant activities without inducing adipogenesis.

Most research groups analyze the vasorelaxant effect of mistletoe preparations by means of isolated aortic ring models and this effect has been observed with triterpenic acids isolated from *Phoradendron reichenbachianum* (Rios et al., 2012). The fact that all preparations or isolated compounds show their relaxant effect on endothelium-dependent manner suggests that nitric oxide synthase (NOS) pathway is involved in the response (Vanhoutte, 2001).

Previous work from our lab determined that *P. calyculatus* aqueous extract had a vasorelaxant effect on precontracted guinea pig aortic rings in a dose-dependent manner. This effect showed to be endothelium dependent, since the effect was abolished by subsequent endothelium removal. It was also determined that this vasorelaxant effect was related to nitric oxide production, for the effect disappeared in the presence of the nitric oxide synthase inhibitor, L-NAME. Therefore, we decided to evaluate the potential vasorelaxant effect from *P. calyculatus* and *Ph. velutinum* by assessing the expression of the *NOS3* gene in cells treated with preparations of both plants. There are reports demonstrating eNOS expression in adipose tissue (Duplain et al., 2001; Nisoli et al., 2003;

Yamada et al., 2015), which is why we chose human subcutaneous adipocytes as our model of study.

P. calyculatus showed an increase of *NOS3* production in freshly induced adipocytes, with a growing effect related to the increasing polarity of the fractions, with the aqueous fraction being the most potent. In terminal adipocytes, HX and WT fractions showed the greater effect on *NOS3* expression. *Ph. velutinum* did not increase *NOS3* expression in induced adipocytes, but CE and WT fraction up-regulated *NOS3* expression in terminal adipocytes.

Antioxidant compounds such as phenolics exert a vasorelaxant effect through the NOS pathway, as shown by a study performed in cultured bovine aortic endothelial cells, where the presence of certain antioxidants produced a three-fold increase in eNOS mRNA (Ramasamy et al., 1999). Another mechanism by which natural compounds may exert their vasorelaxant effect is by increasing eNOS activity, rather than increasing its expression. This was assessed by feeding rats a flavonoid-rich diet and then analyzing NO production in aortic rings. It was determined that the aortic rings of rats fed with either quercetin- or catechin-rich diets showed higher NOS activity, although the expression of eNOS was similar to control group (Benito et al., 2002).

Since aqueous fractions tend to have the most potent biological activities, these results validate the ethnopharmacological use of mistletoe infusions to treat hypertension. Our results also reveal that the bioactive compounds are most likely polar.

Bioactive compounds isolated from several mistletoe species are more often obtained with water or other polar solvents like methanol and ethanol. Various

flavonoids, lignans and phenolic compounds have been identified as principle actives present in mistletoes.

A phytochemical screening of *P. calyculatus* aqueous-methanol extract revealed the presence of gallic acid, catechin, and a quercetin glycoside (Bah et al., 2011). This same research group had already established the vasorelaxant effects of *P. calyculatus* aqueous extract on isolated rat aortic rings (Ibarra-Alvarado et al., 2010). Another phytochemical study demonstrated the presence of tannins, flavonoids and phenylpropanoids in an ethanol extract from the same species (Avila-Acevedo et al., 2012). Said compounds are highly polar due to their chemical structure, which contains several hydroxyl and phenolic groups. Also, quercetin and its glycosylated derivatives are some of the most commonly isolated compounds from several mistletoe species like *Phoradendron californicum, Struthanthus palmeri* (Jiménez-Estrada et al., 2013), *Ligaria cuneifolia* (Fernández et al., 1998; Graziano et al., 1967), *Psittacanthus calyculatus* (Bah et al., 2011), *Tripodanthus acutifolius* (Soberón et al., 2010a; 2010b), *Cladocolea micrantha* (Guimarães et al., 2012), *Tristerix tetrandus* (Simirgiotis et al., 2016), *Arceuthobium* spp. (Crawford and Hawksworth, 1979) and *Phoradendron serotinum* (Alonso-Castro et al., 2013).

Another group of compounds commonly isolated from mistletoe is the terpene group. Terpenes are the most abundant naturally occurring compounds and occur in almost every species (Zhao et al., 2016). Likewise, some terpenic acids such as ursolic, moronic, morolic and betulinic acids have shown a dose-dependent vasorelaxant effect derived from nitric oxide production in rat aortic ring models (Rios et al., 2012). Pentacyclic triterpenoids have wide pharmacological

properties such as anti-oxidant, anti-atherosclerotic, anti-hyperlipidemic (Liu, 2005), cardiotonic and anti-dysrhytmic activities (Senthil et al., 2007; Somova et al., 2003; 2004).

Despite the wide use of *P. calyculatus* and *Phoradendron spp.* in the empirical treatment of type 2 diabetes mellitus, most of their active principles and mechanisms of action remain unknown. A study performed in STZ diabetic rats showed a decrease of glucose levels after 8 h treatment with *P. calyculatus* extract in a dose-dependent manner, an effect that could be due to phenolic compounds (Avila-Acevedo et al., 2012). Similarly, a more profound study used moronic and morolic acids isolated from *Ph. reichenbachianum* to analyze their anti-hyperglycemic and anti-hyperlipidemic effect on an oral glucose tolerance test performed on normoglycemic and diabetic rats. These triterpenes induced significant anti-hyperglycemic effect similar to acarbose, so they proposed two possible mechanisms of action. One of them might involve modulation of insulin action and the other one could be linked to the regulation of glucose uptake from intestinal lumen by delaying carbohydrates digestion, inhibiting enzymes like *α*-glucosidase (Ramírez-Espinosa et al., 2011; 2013).

We decided to evaluate the potential anti-hyperglycemic activity of *P. calyculatus* and *Ph. velutinum* by means of evaluating their insulin-like activity and by assessing their potential *α*-glucosidase inhibitory activity. First by measuring the glucose uptake of human adipocytes in presence of preparations of both plants, using insulin and rosiglitazone as positive control and reference drug, respectively. Second, by determining the potential inhibitory activity on the *α*-glucosidase enzyme.

Both species showed insulin-like activity, by promoting glucose fluorescent analog 2-NBDG incorporation, but the effect was not in a concentration-dependent fashion. As for *α*-glucosidase inhibition, *P. calyculatus* showed *α*-glucosidase inhibition with a minor IC_{50} than acarbose, and the WT fraction resulted the most potent one. *Ph. velutinum* fractions HX and EA exhibited inhibition of *α*-glucosidase, with HX fraction being the most potent one, but only in a high concentration (>1 mg/mL).

Our results suggest that *P. calyculatus* and *Ph. velutinum* exert their hypoglycemic effect by stimulating glucose incorporation by peripheral fat tissue with a similar effect as insulin and rosiglitazone, and by inhibiting the activity of the enzyme *α*-glucosidase.

These results allow us to infer that both species have a notable anti-hyperglycemic effect. This supports the ethnopharmacological use of *P. calyculatus* to treat diabetes, and its WT fraction should be sub-fractioned to reach the specific compounds responsible for this effect. As for *Ph. velutinum*, HX and EA fractions might be suitable for sub-fractionation with the aim of finding active compounds, which could be used inhibitors of *α*-glucosidase and as insulin-like treatments.

Finally, some anti-diabetic drugs increase adipogenesis as a secondary effect, so we wanted to learn if *P. calyculatus* and *Ph. velutinum* exerted pro-adipogenic or anti-adipogenic effects on human preadipocytes. For this purpose, we evaluated the expression of *PPARG*, *CEBPA*, *LEP*, *FABP4* and *KLF4* in induced and terminally differentiated adipocytes.

Adipocyte differentiation is driven by the expression and activation of three transcription factor families: the CAAT/enhancer binding proteins alpha, beta and delta; the helix-loop-helix adipocyte differentiation and determination factor-1; and peroxisome proliferator activated receptor gamma 1 and 2 (Saladin et al., 1999). Since CCAAT/enhancer-binding protein alpha (CEBPα) and peroxisome proliferator-activated receptor gamma (PPARγ) are key regulators during adipogenesis, the down regulation of these genes might prevent the development of obesity. CEBPα and PPARγ expression is induced by CEBPβ and CEBPδ, and together promote differentiation by activating adipose-specific gene expression and by maintaining each other's expression at high levels (Rosen, 2005). Once activated, CEBPα and PPARγ remain elevated for the rest of the differentiation process and for the life of the mature adipocyte (Rosen, 2005) and almost all downstream target genes which define the adipocyte, appear to be regulated by one or both proteins.

Fatty acid-binding protein 4 (FABP4) is a cytoplasmic fatty acid chaperone expressed in adipocytes and implicated in the development of insulin resistance. Either down- or up-regulation of this protein could improve diabetes and obesity, since it is inversely correlated with the expression of PPARγ (Garin-Shkolnik et al., 2014) and it's also one of its targets (Rosen, 2005).

Krüppel-like factor 4 (KLF4) is an essential early regulator of adipogenesis, it functions as an immediate early regulator of adipogenesis to induce CEBPβ (Birsoy et al., 2008).

Leptin (*LEP*) is a hormone that regulates energy intake and expenditure and its actions are mediated by cell-surface receptors found on many different cell

types (Hwang et al., 1997). This hormone regulates food intake and modulates sensitivity to insulin (Stephens, 2012).

Most studies have assessed the anti-adipogenic potential of different plant extracts and isolated natural compounds in the murine cell line 3T3-L1. With such model, several plant extracts have displayed an anti-adipogenic effect by reducing the lipid droplets accumulation and by down-regulating the expression of adipogenic transcription factors (Kim et al., 2004; Shin et al., 2010). In most cases, flavonoids are responsible for the anti-adipogenic effects observed. For instance, quercetin attenuated adipogenesis and decreased expression of adipogenesis-related factors and enzymes in 3T3-L1 cells (Ahn et al., 2008). Other flavonoids like genistein inhibit mitotic clonal expansion, triglyceride accumulation and *PPARG* expression (Harmon and Harp, 2001). Rutin is also able to down-regulate the expression of *PPARG* and *CEBPA* in 3T3-L1 cells (Choi et al., 2006). Apigenin, showed a suppression on adipocyte differentiation of 3T3-L1 cells by decreasing the levels of *PPARG* and one of its target genes, *FABP4*; and a reduction in the accumulation of intracellular lipids (Ono and Fujimori, 2011).

Even though 3T3-L1 cells is an extensively used model, there are almost no studies performed on human adipocytes, which is why we decided to use human adipocytes to get a better insight to the biological effects exerted by the mistletoe species we analyzed.

Our results show that *P. calyculatus* CE exerts the greatest anti-adipogenic effect by attenuating the expression of adipogenic genes during the induction period, and the fractions show a differential effect on each gene, suggesting that compounds with different polarity might be responsible for inhibiting the expression

of each gene, displaying a synergistic effect when acting altogether. In terminal adipocytes, adipogenic genes expression is not significantly affected by the presence of any of the fractions, with the exception of *PPARG* and *CEBPA*, which are still down-regulated in presence of CE and to a lesser extent, in presence of EA fraction. On the contrary, all fractions increased *KLF4* expression in a dose-dependent manner. This could be due to the fact that, besides being an early key regulator of adipogenesis, KLF4 is also a critical regulator of endothelial homeostasis, and the application of vasoprotective stimuli, induces the expression of *KLF4* in culture human endothelial cells (Villareal Jr et al., 2009). As we are applying potential vasorelaxant extracts and fractions, this could explain the up-regulation of *KLF4* in terminal adipocytes.

As for *Ph. velutinum*, CE and the least polar fractions down-regulated the expression of *PPARG* and *LEP* in induced adipocytes as much as or even more than the negative control, retinoic acid. All fractions except WT down-regulated *FABP4* expression in a dose-dependent manner. *CEBPA* was only mildly down-regulated by CE and fractions, and only WT fraction in the highest concentration showed a visible inhibitory effect. In terminal adipocytes, CE, EA and WT fractions reduced the expression of *PPARG* and *FABP4* in a dose-dependent manner. EA and WT fractions reduced the expression of *CEBPA* in a dose-dependent manner, and only WT fraction reduced the expression of *LEP*. None of the fractions had an inhibitory effect on the expression of *KLF4*, and only HX fraction increased the expression of this gene. This results suggest that, the bioactive compounds with anti-adipogenic potential are comprised in the medium polarity and high polarity fractions of *Ph. velutinum*.

PPARγ induces the expression of *CEBPA* and then binds with CEBPα to the promoter of *FABP4*. Thus, the down-regulation of either *PPARG* or *CEBPA* could result in a lower expression of *FABP4* (Rosen, 2005). Inversely, FABP4 negatively regulates PPARγ levels in adipocytes, thereby attenuating adipocyte differentiation (Garin-Shkolnik et al., 2014). Then, it is possible that *FABP4* expression is reduced because at least one of the two most important key regulators of adipogenesis (PPARγ and CEBPα) is down-regulated by mistletoe extracts.

Obesity is a risk factor for developing type 2 diabetes and cardiovascular disease, which allowed us to correlate our results. The extracts and fractions of *P. calyculatus* and *Ph. velutinum* induced eNOS expression in adipocytes, and eNOS in adipocytes possesses an anti-lipolytic action (Yamada et al., 2015). Moreover, PPARγ negatively regulates eNOS expression in adipocytes (Yamada et al., 2015), which concords with our results, as we observed an increase in *NOS3* expression and a decrease in *PPARG* expression. Nitric oxide increases energy expenditure and regulates glucose and lipid metabolism (Sansbury and Hill, 2014). Thus, by overexpressing eNOS in adipocytes, it is possible to prevent diet-induced obesity, increase whole body metabolism, improve diet-induced insulin resistance and prevent adipocyte hypertrophy and size dispersion (Sansbury et al., 2012)

In conclusion, our results show diverse biological effects of *P. calyculatus* and *Ph. velutinum*, since *P. calyculatus* demonstrated a better anti-hyperglycemic effect than *Ph. vellutinum*. Both plants have a potential anti-hypertensive effect by means of up-regulating the expression of *NOS3*, with the polar fractions being the most potent, and both species possess anti-adipogenic activities. *P. calyculatus* anti-adipogenic activity is stronger when cells are treated with crude extract,

probably due to a synergistic effect of compounds with different polarity. *Ph. velutinum* exerts a greater anti-adipogenic when cells are in the presence of WT and EA fractions, the ones which are more abundant in CE, indicating that this effect is exerted by medium and high polarity compounds.

Our perspectives comprise the isolation and structural characterization of bioactive compounds that can be responsible for the observed biological effects and the evaluation of such compounds directly on eNOS activity. Correspondingly, it is essential to deepen into the insulin-like activity of these preparations. Further work will also require the evaluation of bioactive compounds with *in vivo* models and to perform acute and chronic toxicity assays. With this, it would be possible not only the identification of new and more effective drugs of natural origin for the treatment of the metabolic syndrome and their specific mechanisms of action, but also the reaching of a deeper knowledge of the cell function.

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Figure 1. Analytic HPLC-UV chromatogram of WT fraction from *Ph. velutinum.* WT fraction from *Ph. velutinum* was dissolved in MeOH at a 0.5 mg/mL concentration and submitted to HPLC-UV analysis as described in Materials and methods. Parameters: Purospher STAR RP-18 column (Merckmillipore, 4.5 x 250 mm, 5 μm), isocratic elution (MeOH/acetic acid in H_2O 0.5% in a 60:40 relation), wavelength detection: 254 nm, injection volume: 20 μL, flow: 0.4 mL/min.

Figure 2. ESIMS$^+$ analysis of the crude WT fraction from *Ph. vellutinum.* WT fraction from *Ph. velutinum* was dissolved in MeOH at a 1 mg/mL concentration and submitted to ESIMS analysis as described in Materials and methods. The data were acquired in mode of positive detection in a Bruker Daltonics Squire 6000 Mass Spectrometer.

Figure 3. ^{1}H NMR spectrum of the crude WT fraction from *Ph. vellutinum.* WT fraction from was prepared as described in Materials and methods. NMR data were acquired on a Bruker Avance III Spectrometer at 400 MHz.

Figure 4. ESIMS/MS$^+$ spectrum of the molecular ion at *m/z* 485 $[M + H]^+$. WT fraction from *Ph. velutinum* was dissolved in MeOH at a 1 mg/mL concentration and submitted to ESIMS analysis as described in Materials and methods. The data were acquired in mode of positive detection in a Bruker Daltonics Squire 6000 Mass Spectrometer.

Figure 5. ESIMS/MS$^+$ spectrum of the molecular ion at *m/z* 469 $[M + H]^+$. WT fraction from *Ph. velutinum* was dissolved in MeOH at a 1 mg/mL concentration and submitted to ESIMS analysis as described in Materials and methods. The data were acquired in mode of positive detection in a Bruker Daltonics Squire 6000 Mass Spectrometer.

Figure 6. Cell viability of human subcutaneous preadipocytes cultured in the presence of *P. calyculatus* and *Ph. velutinum* preparations. Normal human

subcutaneous preadipocytes were seeded (5 × 10^3 cells/well) in 96 well microplates with basal medium (BM). After 24 h, the cells were treated with BM and the indicated concentrations of *P. calyculatus* (A) or *Ph. velutinum* (B) preparations for 48 h. Then, cell survival was evaluated by MTT assay. (0.01 μg/mL, 0.1 μg/mL, 1 μg/mL, 10 μg/mL, 50 μg/mL).

Figure 7. Effect of *P. calyculatus* and *Ph. velutinum* on the expression of *NOS3* in cultured human adipocytes. Human subcutaneous preadipocytes were induced to differentiation with MDI and the indicated concentrations of crude extract (CE), hexane (HX), ethyl acetate (EA) and aqueous (WT) fractions of *P. calyculatus* (A, B) and *Ph. vellutinum* (C, D) as described in Materials and methods. Total RNA was recovered from freshly induced (A, C) or terminally differentiated (B, D) adipocytes and analyzed by RT-PCR using specific primers for *NOS3* mRNA. (10 μg/mL, 30 μg/mL).

Figure 8. Effect of *Psittacanthus calyculatus* preparations on gene expression of freshly induced human adipocytes. Human subcutaneous preadipocytes were induced to differentiation with MDI and the indicated concentrations of crude extract (CE), hexane (HX), ethyl acetate (EA) and aqueous (WT) fractions of *P. calyculatus* as described in Materials and methods section. After 6 days, total RNA was extracted from treated cells and analyzed by RT-PCR using specific primers for the indicated genes. (10 μg/mL, 30 μg/mL).

Figure 9. Effect of *Psittacanthus calyculatus* preparations on gene expression of terminally differentiated human adipocytes. Human subcutaneous preadipocytes were induced to differentiation with MDI and the indicated concentrations of crude extract (CE), hexane (HX), ethyl acetate (EA) and aqueous (WT) fractions of *P. calyculatus* as described in Materials and methods section. After 17 days, total RNA was extracted from treated cells and analyzed by RT-PCR using specific primers for the indicated genes. (10 μg/mL, 30 μg/mL).

Figure 10. Effect of *Phoradendron velutinum* preparations on gene expression of freshly induced human adipocytes. Human subcutaneous preadipocytes were induced to differentiation with MDI and the indicated concentrations of crude extract (CE), hexane (HX), ethyl acetate (EA) and aqueous (WT) fractions of *Ph. velutinum* as described in Materials and methods section. After 6 days, total RNA was extracted from treated cells and analyzed by RT-PCR using specific primers for the indicated genes. (10 µg/mL, 30 µg/mL).

Figure 11. Effect of *Phoradendron velutinum* preparations on gene expression of terminally differentiated human adipocytes. Human subcutaneous preadipocytes were induced to differentiation with MDI and the indicated concentrations of crude extract (CE), hexane (HX), ethyl acetate (EA) and aqueous (WT) fractions of *Ph. velutinum* as described in Materials and methods section. After 17 days, total RNA was extracted from treated cells and analyzed by RT-PCR using specific primers for the indicated genes. (10 µg/mL, 30 µg/mL).

Figure 12. Effects of *P. calyculatus* and *Ph. velutinum* preparations on 2-NBDG uptake in normal human adipocytes. hASCB1 adipocytes were incubated in PBS 0.1% BSA with preparations (10 µg/mL or 30 µg/mL) from either *P. calyculatus* (A) or *Ph. vellutinum* (B), 100 nM insulin or 10 µM RGZ with 80 µM 2-NBDG for 1 h. Absorbed 2-NBDG was measured with a microplate reader (10 µg/mL, 30 µg/mL).

Figure 13. *α*-Glucosidase inhibition of *P. calyculatus* and *Ph. velutinum* preparations. Reaction mixtures for *α*-glucosidase activity were added with different concentrations of *P. calyculatus* (A) and *Ph. velutinum* (B) preparations as described in Materials and methods. The curves show the increase of enzyme inhibitory activity exerted by the treatments according to their concentration.

Table 1. Extracts and fractions of American mistletoes.

Species	Material (g)	Extract[1] (g)	Yield (%)	Fractions	Weight (g)	Yield (%)
Psittacanthus calyculatus	1,500	247.78	16	HX[2]	161.06	65
				EA[3]	10.9	4.4
				WT[4]	61.9	25
Phoradendron velutinum	471.5	160.44	34	HX[2]	9.87	6
				EA[3]	28.19	17
				WT[4]	22.38	14

[1]Obtained with a mixture of dichloromethane:methanol 1:1
[2]Hexane
[3]Ethyl acetate
[4]Water

Table 2. Primers sequences for RT-PCR.

mRNA	Forward sequence Reverse sequence	Amplicon (bp)	T_a (°C)
GAPDH	5'GAAGGTGGTGAAGCAGGCGT3' 5'ATGTGGGCCATGAGGTCCACCA3'	216	62
FABP4	5'CAGTGTGAATGGGGATGTGA3' 5'GTGGAAGTGACGCCTTTC3'	249	61
KLF4	5'CTGCTCCCATCTTTCTCCAC3' 5'GTTGAACTCCTCGGTCTCTC3'	200	60.5
PPARG	5'ATGGGTGAAACTCTGGGAGA3' 5'TGGAATGTCTTCGTAATGTGGA3'	246	57
CEBPA	5'ACTGAGTAGGGGGAGCAAATCGTG3' 5'GTTCCAAGCCCCAAGTCCCTATG3'	220	57
LEP	5'ACACGCAGTCAGTCTCCT3' 5'AGGTTCTCCAGGTCGTTG3'	172	57
NOS3	5'CATCACCAGGAAGAAGACCTTA3' 5'TACAGGATTGTCGCCTTCAC3'	105	58

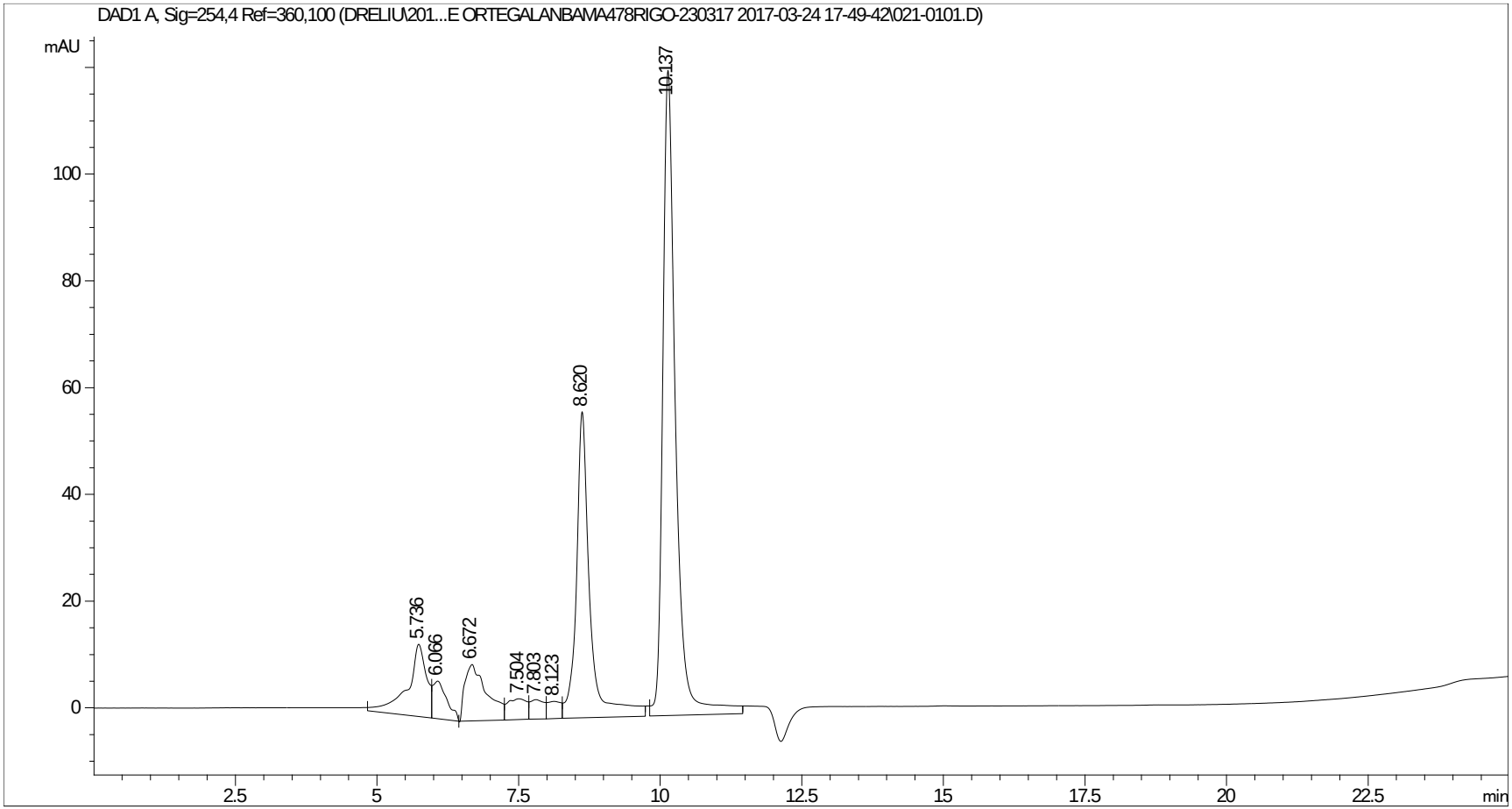
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Figure 1.

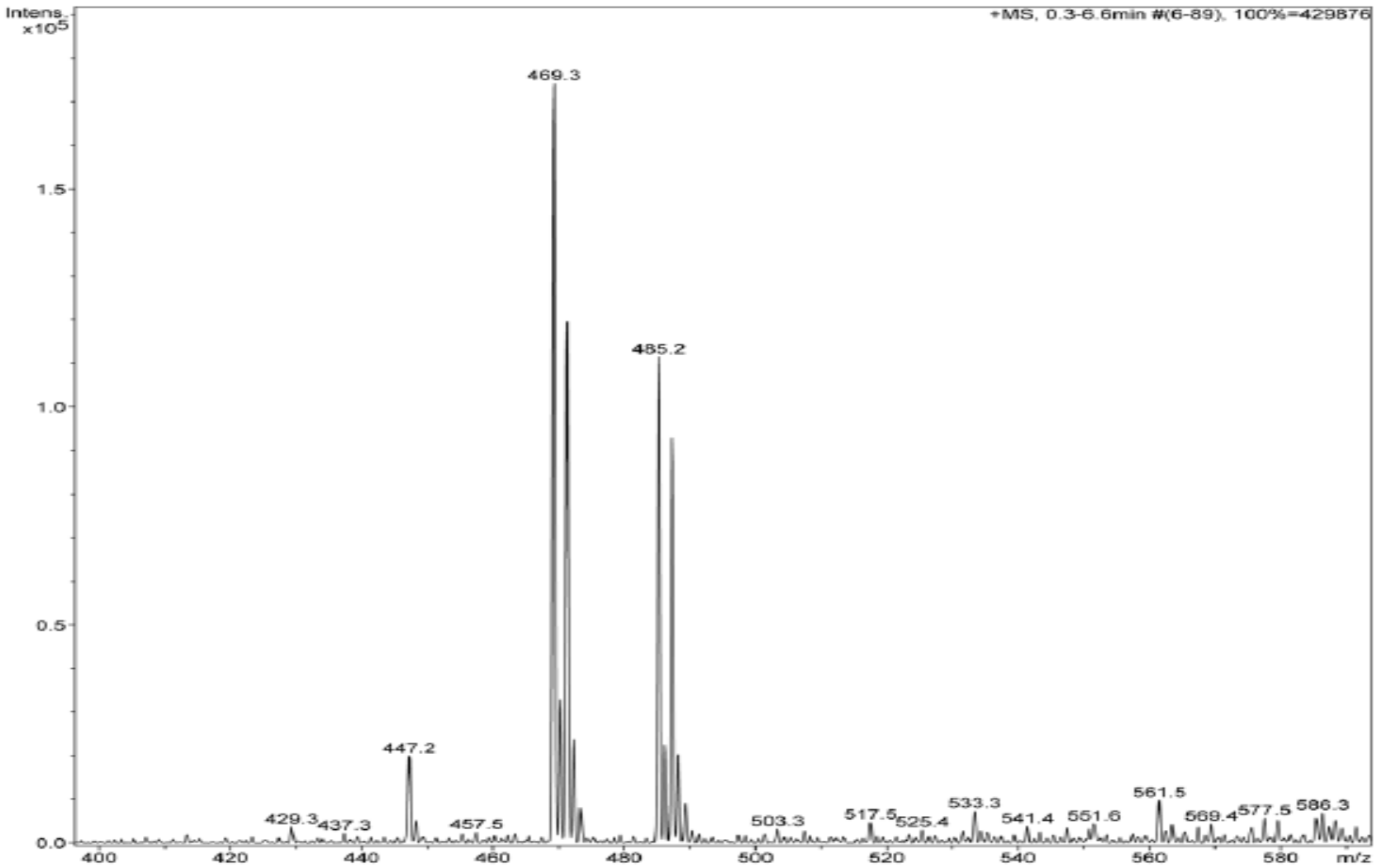
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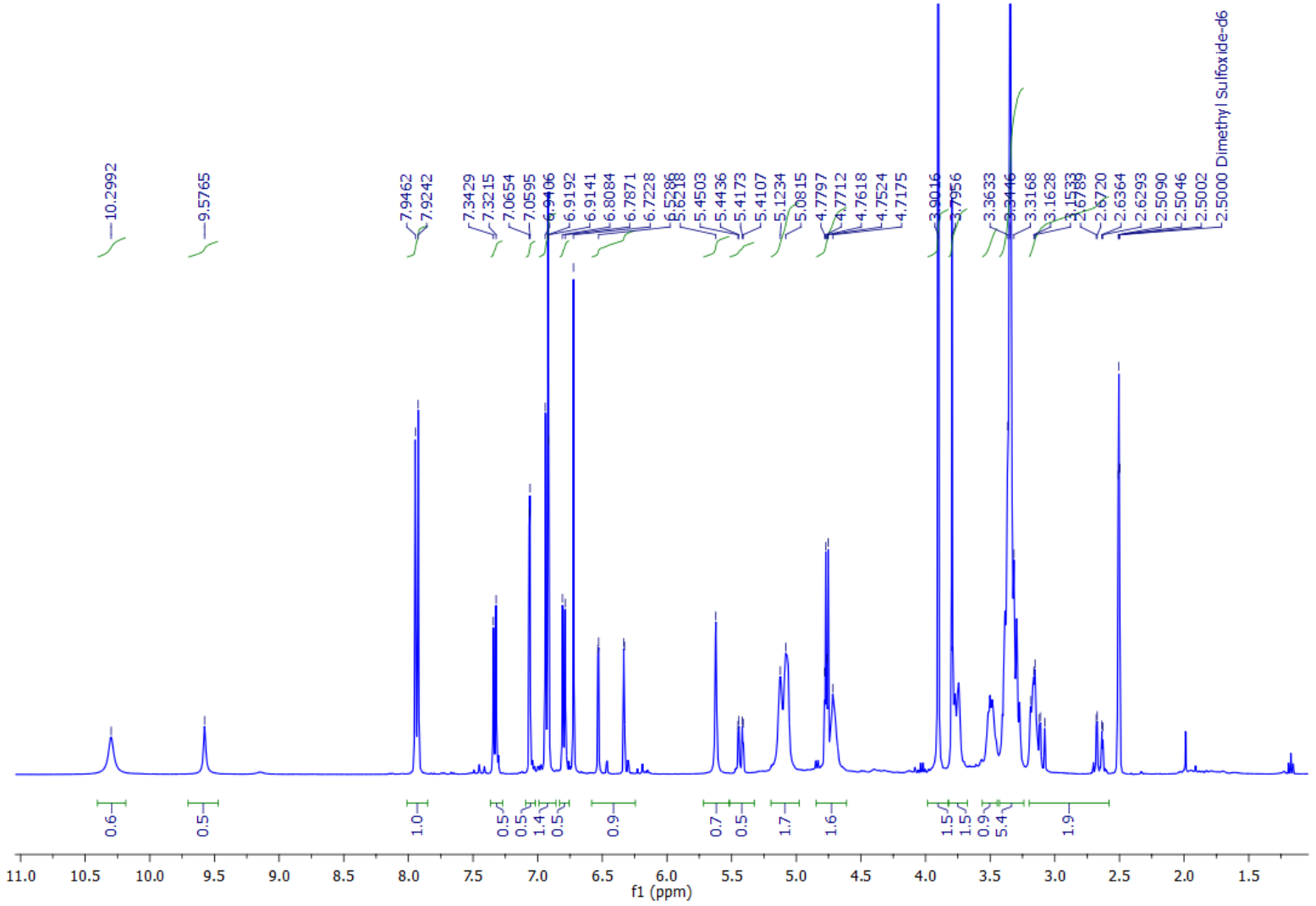
Figure 2.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f1 (ppm)

Figure 3.

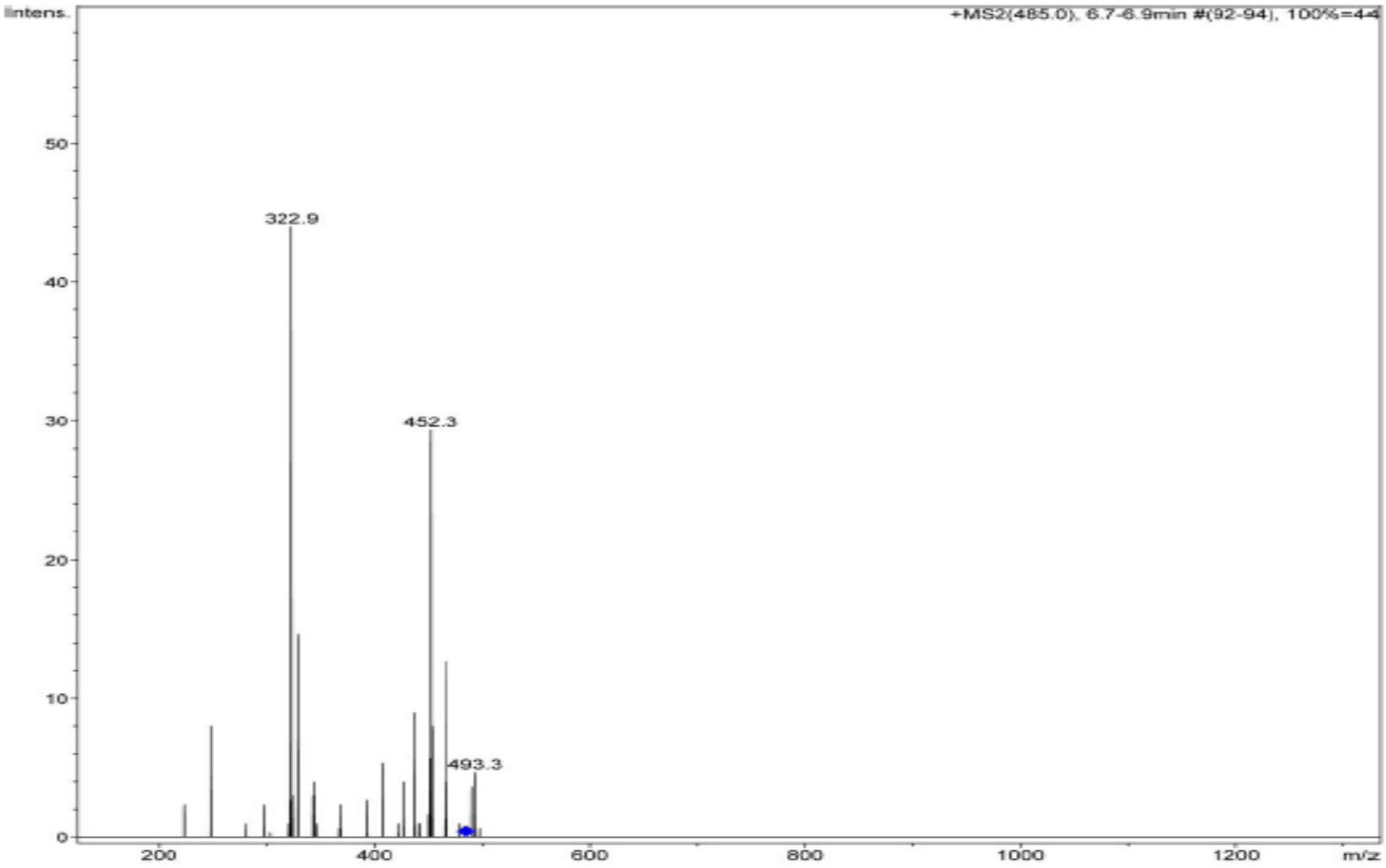
Intens.
+MS2(485.0), 6.7-6.9min #(92-94), 100%=44
50
40
30
20
10
0
322.9
452.3
493.3
200
400
600
800
1000
1200
m/z

Figure 4.

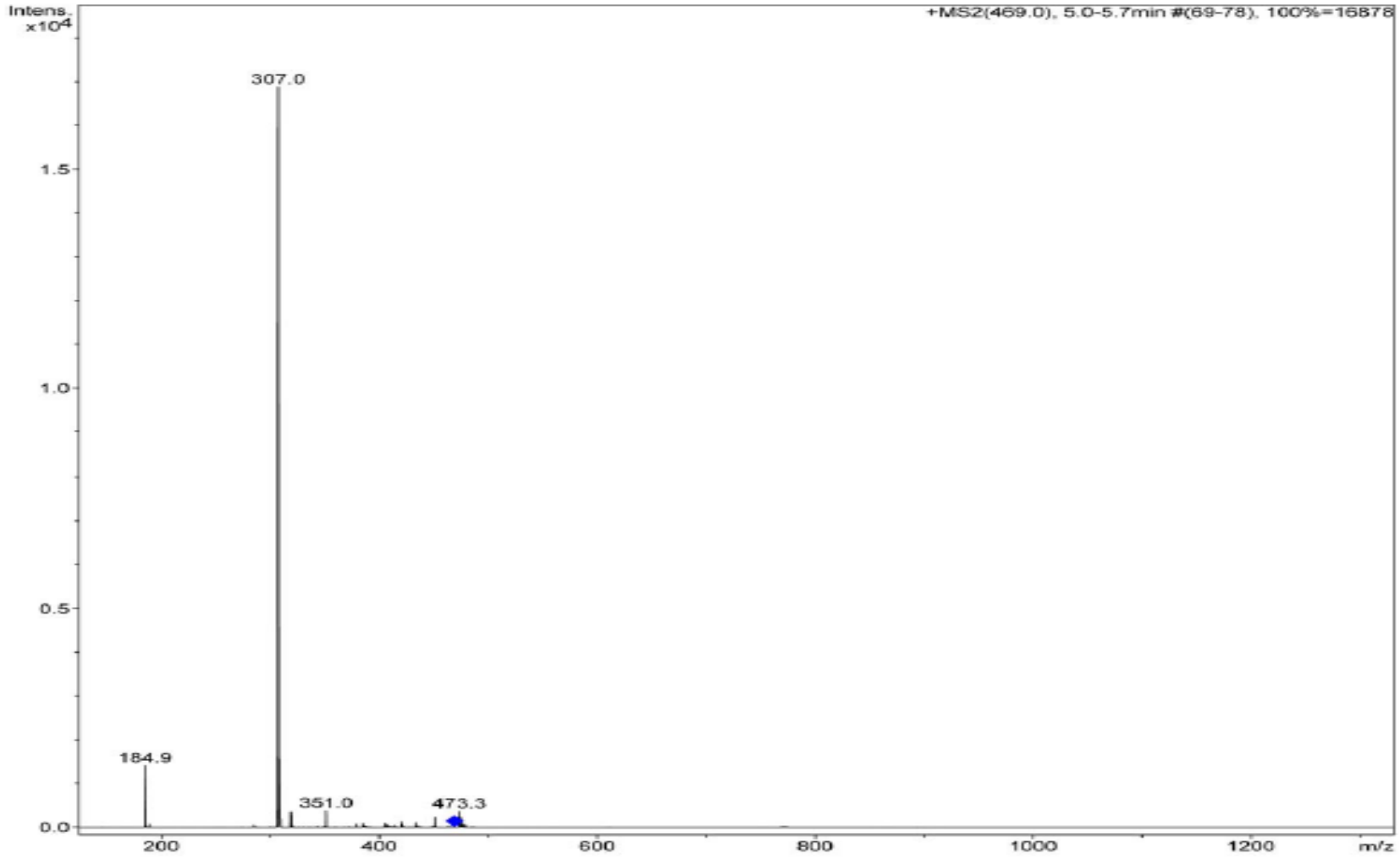
Intens.
x10^4
+MS2(469.0), 5.0-5.7min #(69-78), 100%=16878
307.0
1.5
1.0
0.5
0.0
184.9
351.0
473.3
200
400
600
800
1000
1200
m/z

Figure 5.

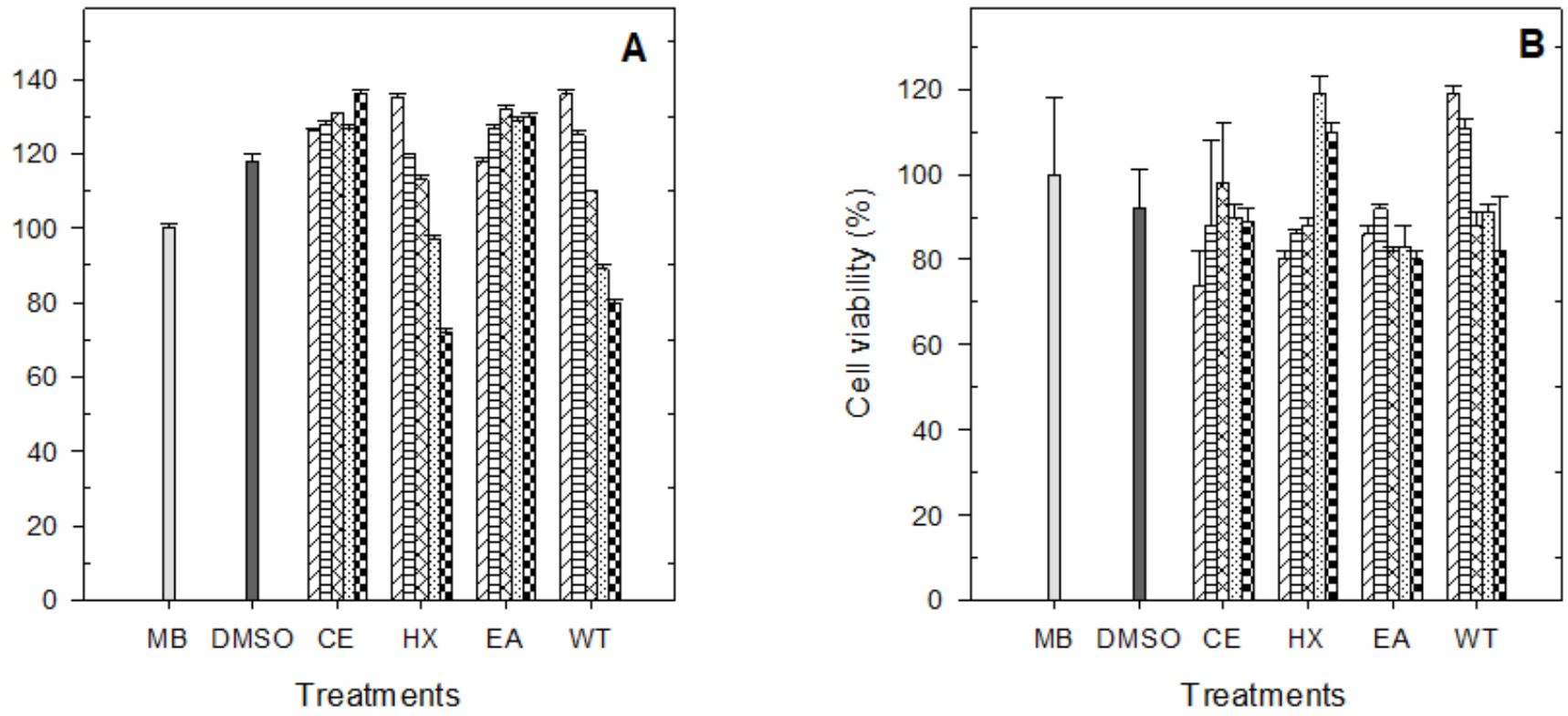
A
140
120
100
80
60
40
20
0
MB
DMSO
CE
HX
EA
WT
Treatments
B
Cell viability (%)
120
100
80
60
40
20
0
MB
DMSO
CE
HX
EA
WT
Treatments

Figure 6.

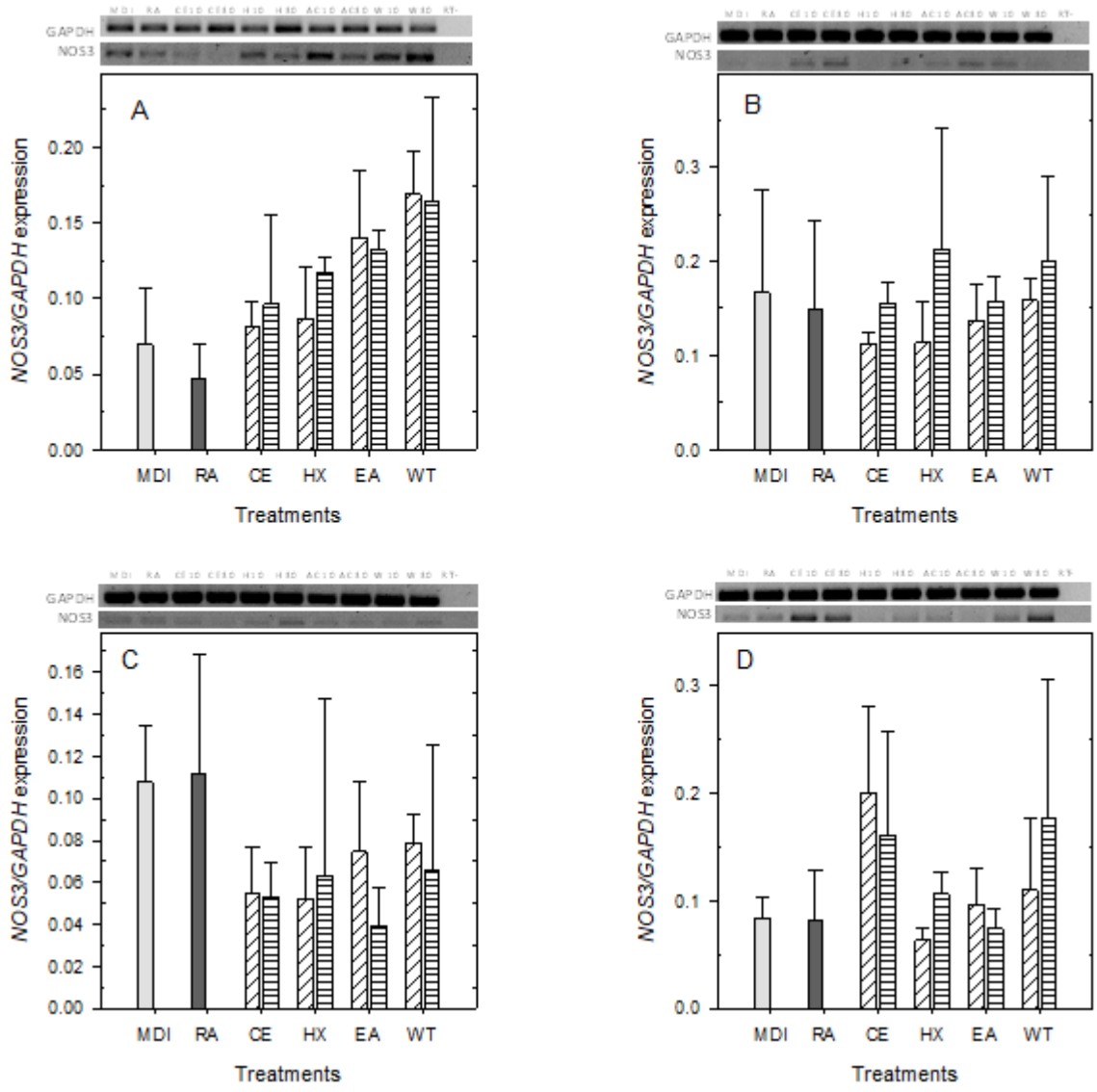
GAPDH
NOS3
A
NOS3/GAPDH expression
0.00
0.05
0.10
0.15
0.20
MDI
RA
CE
HX
EA
WT
Treatments
B
0.0
0.1
0.2
0.3
C
0.00
0.02
0.04
0.06
0.08
0.10
0.12
0.14
0.16
D

Figure 7.

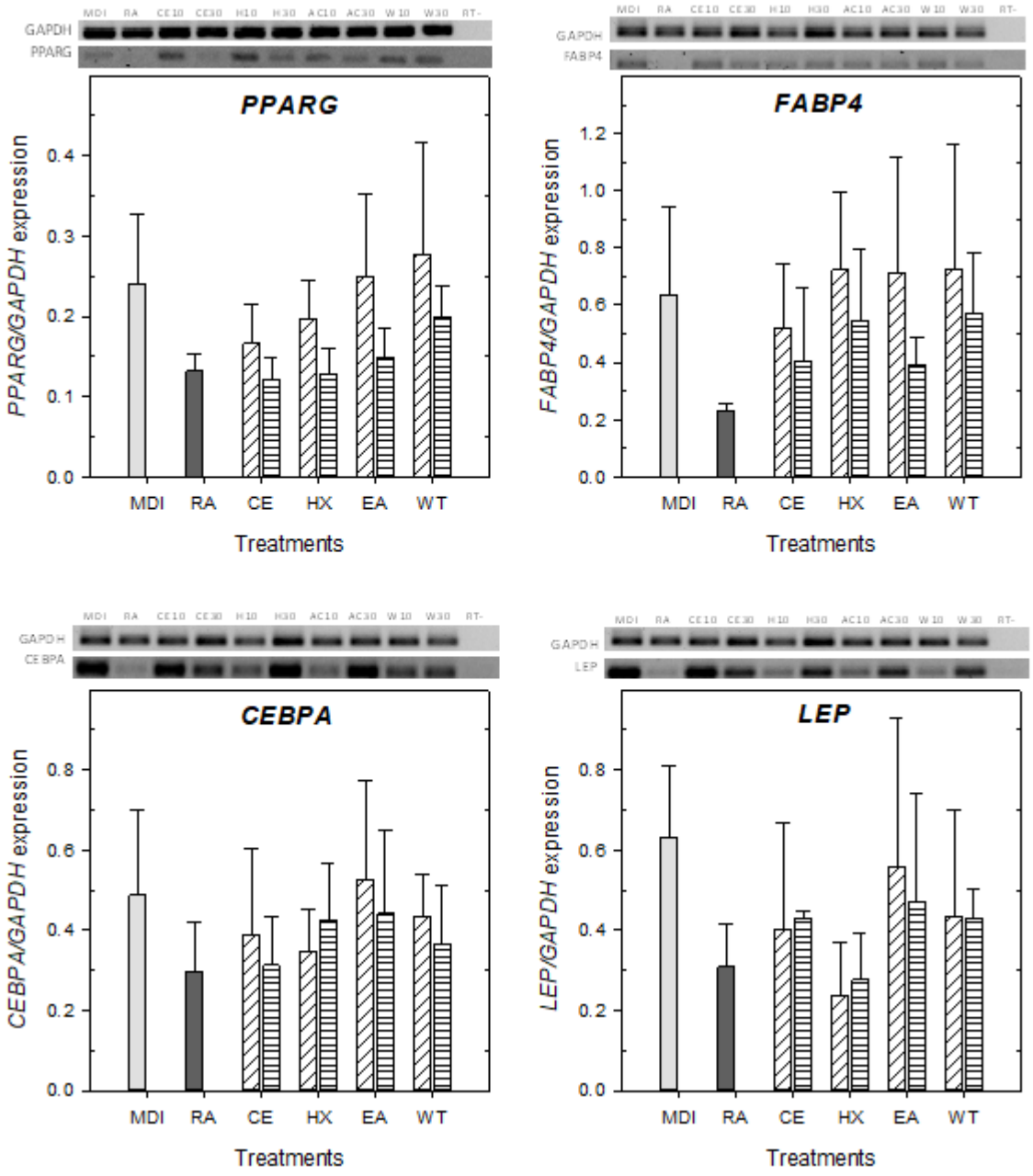
MDI RA CE10 CE30 H10 H30 AC10 AC30 W10 W30 RT-
GAPDH
PPARG
PPARG
PPARG/GAPDH expression
0.0 0.1 0.2 0.3 0.4
MDI RA CE HX EA WT
Treatments
GAPDH
FABP4
FABP4
FABP4/GAPDH expression
0.0 0.2 0.4 0.6 0.8 1.0 1.2
MDI RA CE HX EA WT
Treatments
GAPDH
CEBPA
CEBPA
CEBPA/GAPDH expression
0.0 0.2 0.4 0.6 0.8
MDI RA CE HX EA WT
Treatments
GAPDH
LEP
LEP
LEP/GAPDH expression
0.0 0.2 0.4 0.6 0.8
MDI RA CE HX EA WT
Treatments

Figure 8.

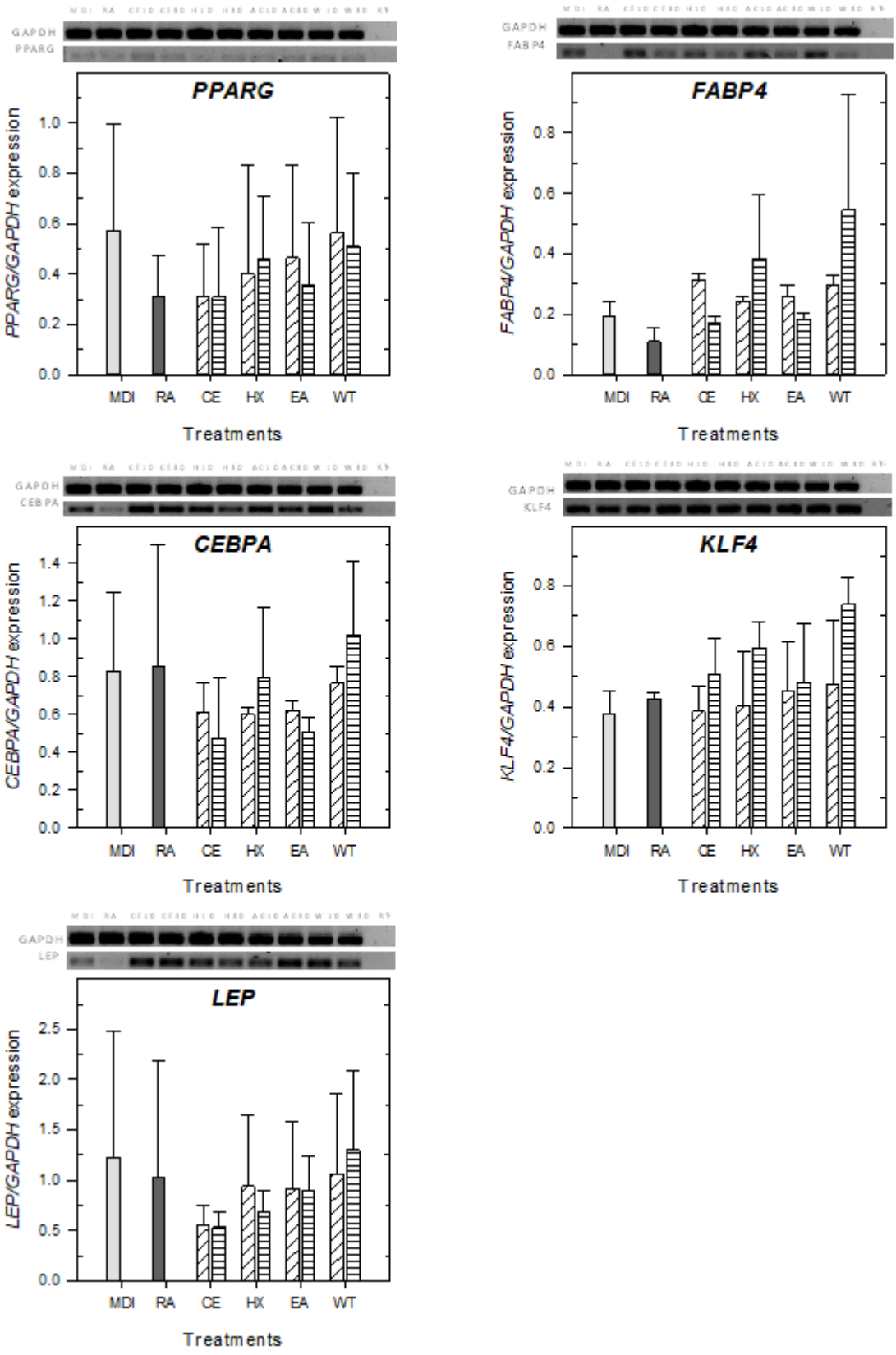
GAPDH
PPARG
PPARG
PPARG/GAPDH expression
0.0
0.2
0.4
0.6
0.8
1.0
MDI
RA
CE
HX
EA
WT
Treatments
GAPDH
FABP4
FABP4
FABP4/GAPDH expression
0.0
0.2
0.4
0.6
0.8
MDI
RA
CE
HX
EA
WT
Treatments
GAPDH
CEBPA
CEBPA
CEBPA/GAPDH expression
0.0
0.2
0.4
0.6
0.8
1.0
1.2
1.4
MDI
RA
CE
HX
EA
WT
Treatments
GAPDH
KLF4
KLF4
KLF4/GAPDH expression
0.0
0.2
0.4
0.6
0.8
MDI
RA
CE
HX
EA
WT
Treatments
GAPDH
LEP
LEP
LEP/GAPDH expression
0.0
0.5
1.0
1.5
2.0
2.5
MDI
RA
CE
HX
EA
WT
Treatments

Figure 9.

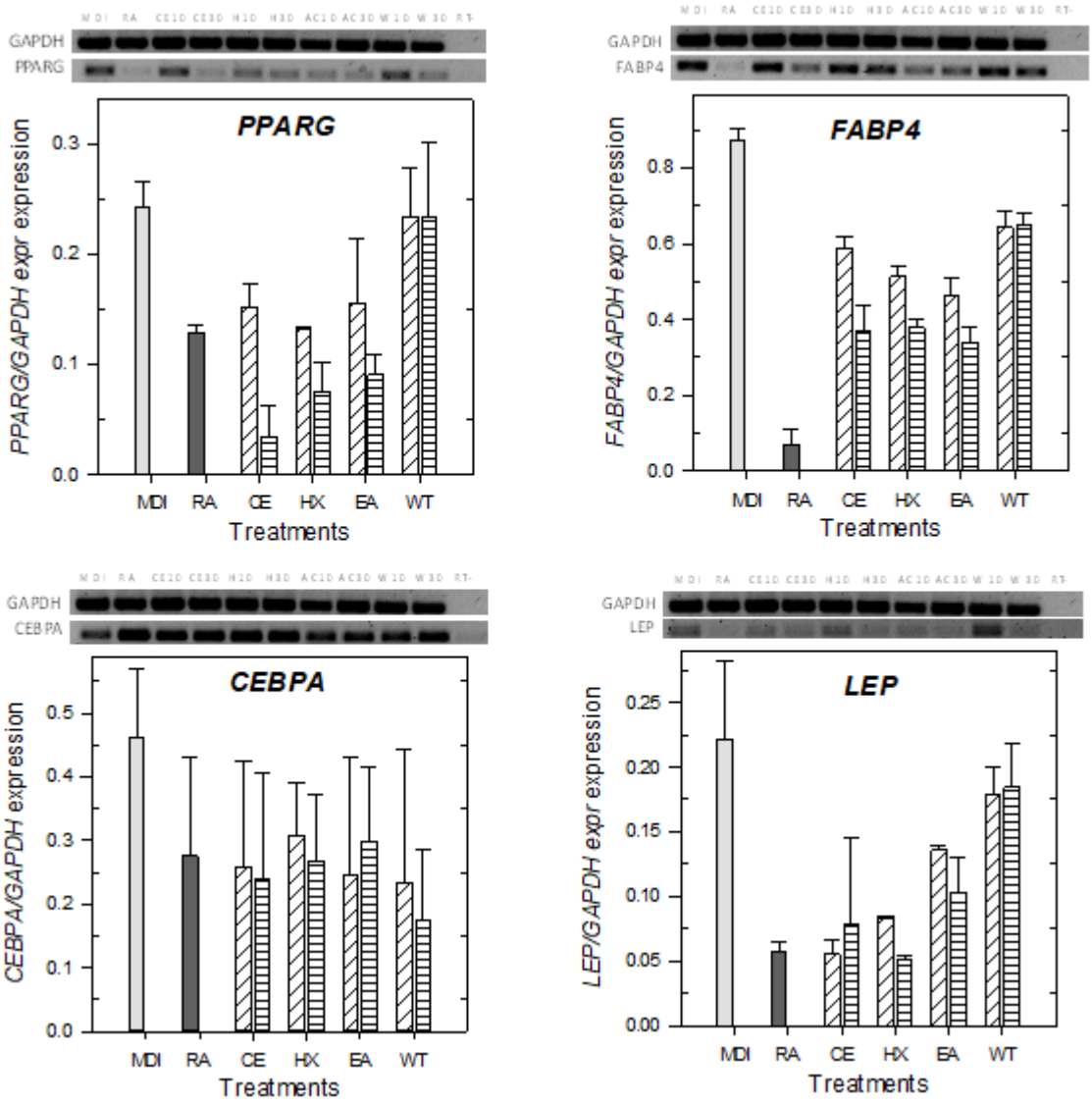
GAPDH
PPARG
PPARG
PPARG/GAPDH expr expression
0.0
0.1
0.2
0.3
MDI
RA
CE
HX
EA
WT
Treatments
GAPDH
FABP4
FABP4
FABP4/GAPDH expr expression
0.0
0.2
0.4
0.6
0.8
MDI
RA
CE
HX
EA
WT
Treatments
GAPDH
CEBPA
CEBPA
CEBPA/GAPDH expression
0.0
0.1
0.2
0.3
0.4
0.5
MDI
RA
CE
HX
EA
WT
Treatments
GAPDH
LEP
LEP
LEP/GAPDH expr expression
0.00
0.05
0.10
0.15
0.20
0.25
MDI
RA
CE
HX
EA
WT
Treatments

Figure 10.

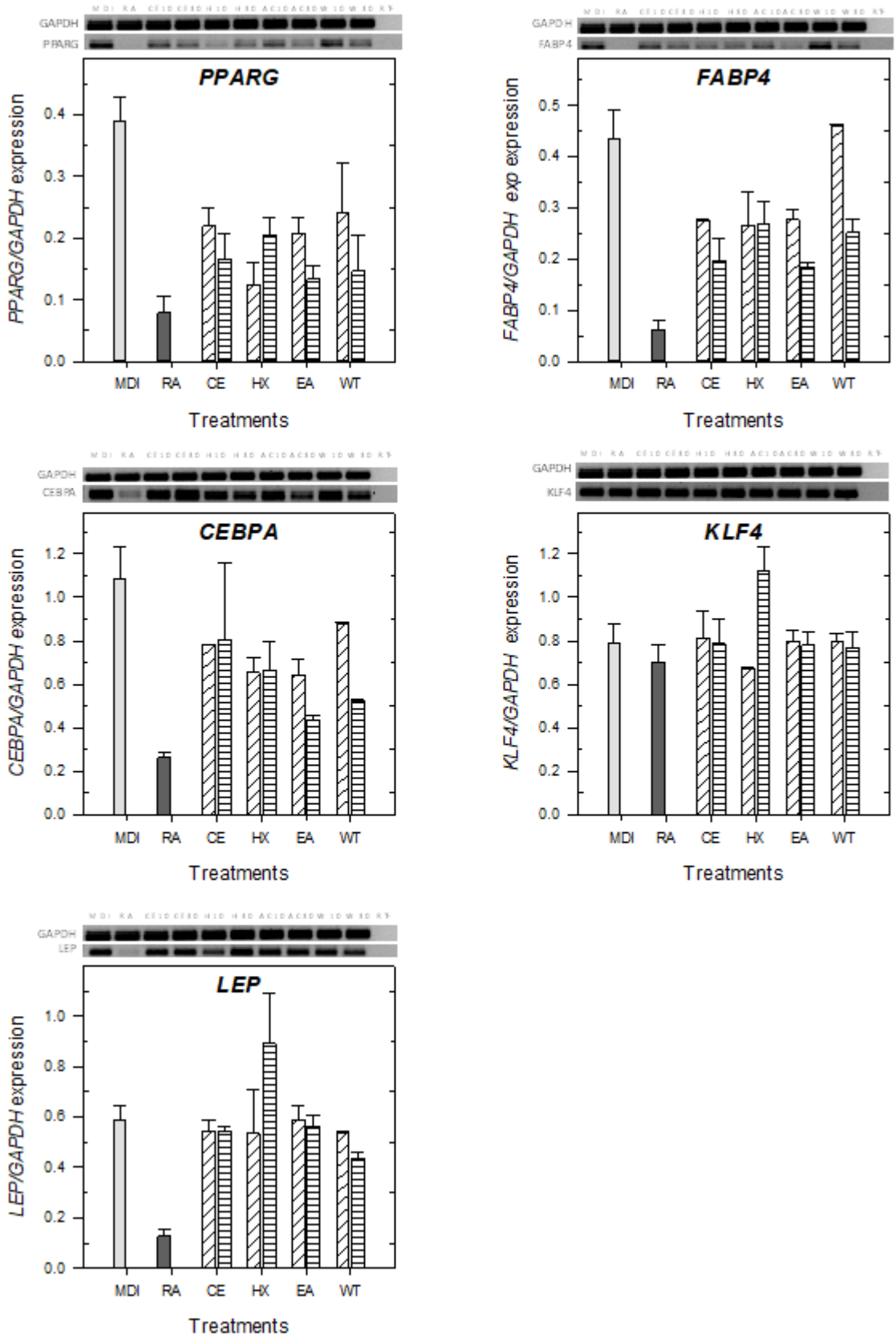
GAPDH
PPARG
PPARG
PPARG/GAPDH expression
0.0
0.1
0.2
0.3
0.4
MDI
RA
CE
HX
EA
WT
Treatments
GAPDH
FABP4
FABP4
FABP4/GAPDH exp expression
0.0
0.1
0.2
0.3
0.4
0.5
MDI
RA
CE
HX
EA
WT
Treatments
GAPDH
CEBPA
CEBPA
CEBPA/GAPDH expression
0.0
0.2
0.4
0.6
0.8
1.0
1.2
MDI
RA
CE
HX
EA
WT
Treatments
GAPDH
KLF4
KLF4
KLF4/GAPDH expression
0.0
0.2
0.4
0.6
0.8
1.0
1.2
MDI
RA
CE
HX
EA
WT
Treatments
GAPDH
LEP
LEP
LEP/GAPDH expression
0.0
0.2
0.4
0.6
0.8
1.0
MDI
RA
CE
HX
EA
WT
Treatments

Figure 11.

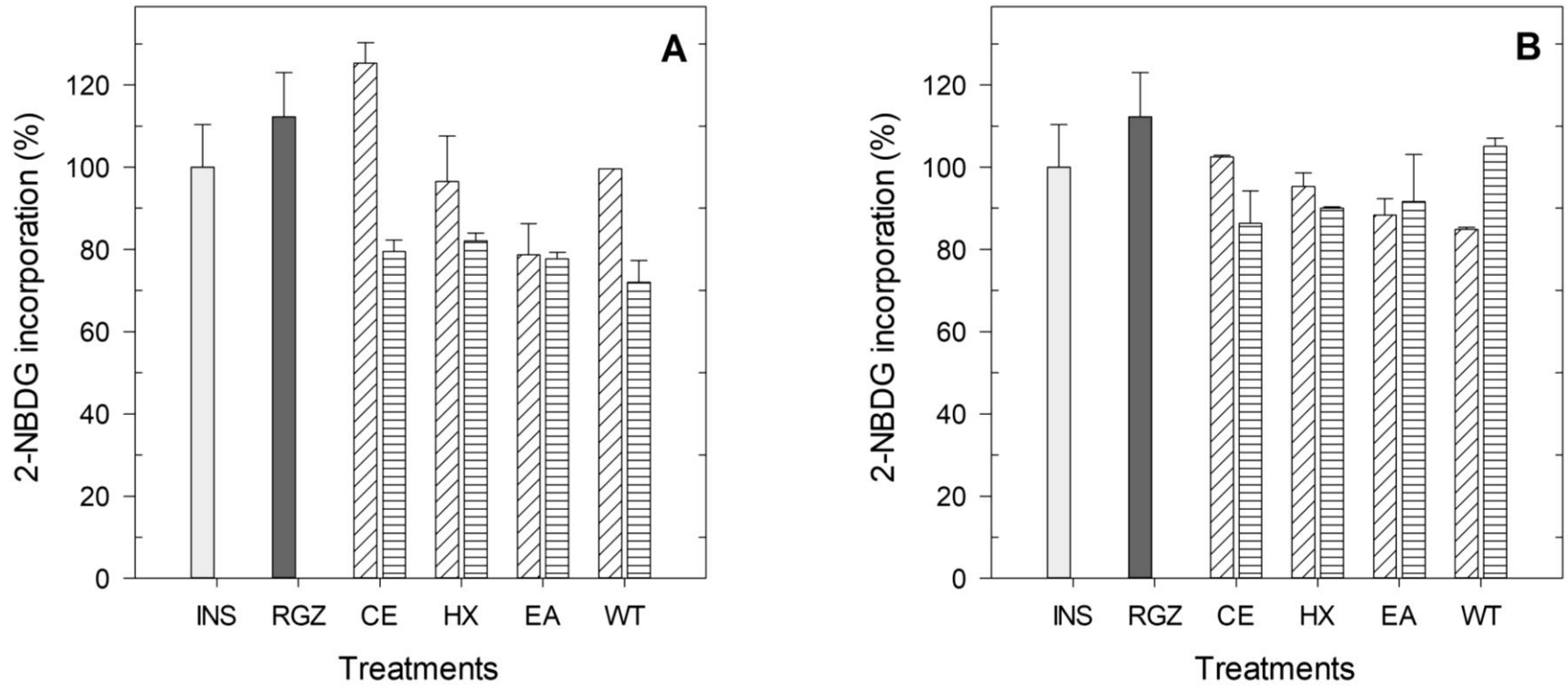
A
B
2-NBDG incorporation (%)
0
20
40
60
80
100
120
INS
RGZ
CE
HX
EA
WT
Treatments

Figure 12.

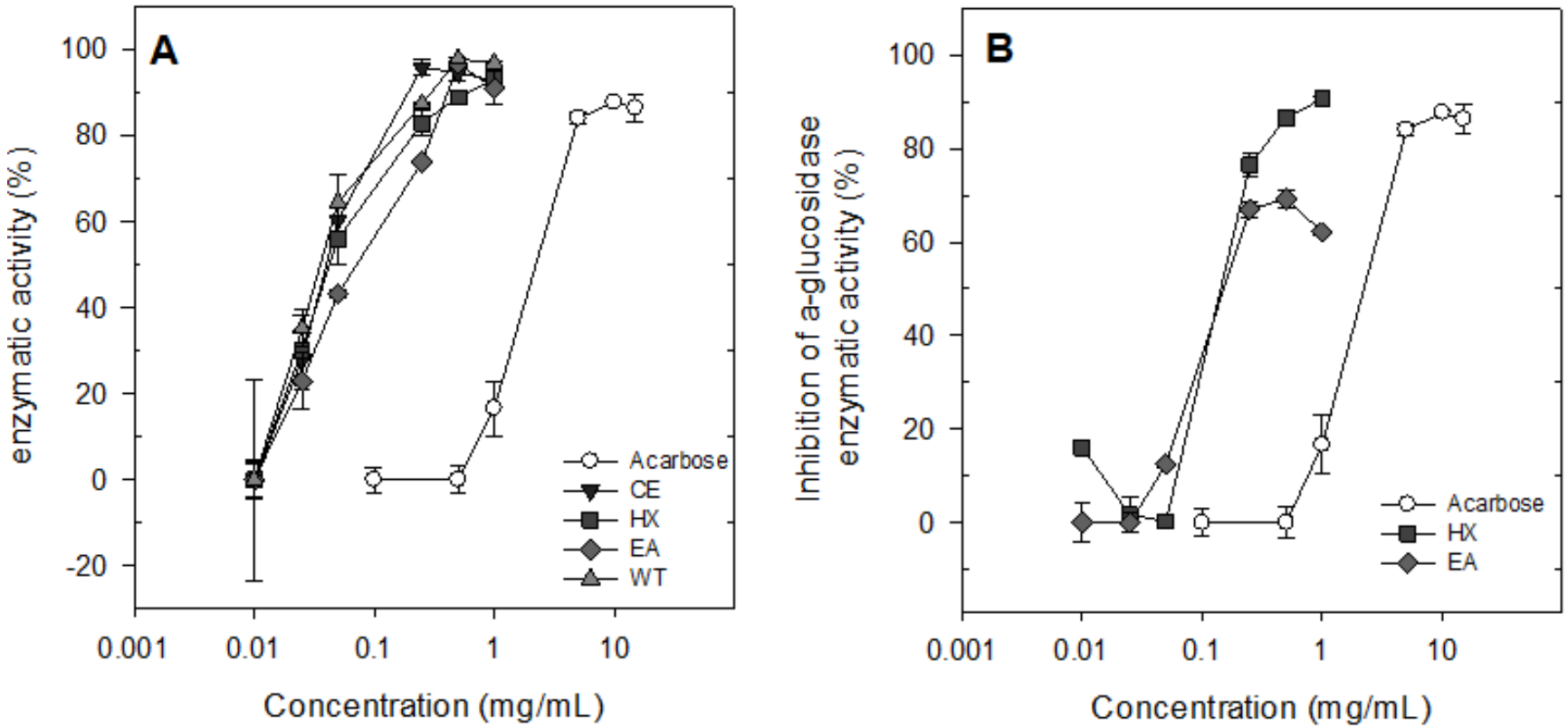
A
100
80
60
40
20
0
-20
enzymatic activity (%)
0.001
0.01
0.1
1
10
Concentration (mg/mL)
Acarbose
CE
HX
EA
WT
B
100
80
60
40
20
0
Inhibition of a-glucosidase enzymatic activity (%)
0.001
0.01
0.1
1
10
Concentration (mg/mL)
Acarbose
HX
EA

Figure 13.