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Original article

Turnera diffusa extract attenuates profibrotic, extracellular matrix and mitochondrial markers in activated human hepatic stellate cells (HSC)

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ABSTRACT

Introduction and objectives: Hepatic fibrosis is characterized by the accumulation of extracellular matrix which includes the accumulation of α-smooth muscle actin (α-SMA), collagen type I (COL1α1), as well as remodeling induced by metalloproteinases and tissue inhibitor of metalloproteinase (TIMPs), where hepatic stellate cells (HSCs) play a central role. In addition, the transcription factor SNAI1 (which participates in epithelial-mesenchymal transition, EMT) and mitofusin 2 (MFN2, a mitochondrial marker) plays an important role in chronic liver disease. *Turnera diffusa* (TD), a Mexican endemic plant, has been shown to possess antioxidant and hepatoprotective activity *in vitro*. We treated human HSC (LX2 cells) with a methanolic extract of *Turnera diffusa* (METD) to evaluate the mechanism involved in its hepatoprotective effect measured as fibrosis modulation, EMT, and mitochondrial markers.
Materials and methods: HSC LX-2 cells were treated with METD (100 and 200 ng/mL) alone or combined with TGF-β (10 ng/mL) at different time points (24, 48, and 72 h). *α-SMA, COL1α1, MMP2, TIMP1, SNAI1,* and *MFN2* mRNAs and protein levels were determined by real-time quantitative PCR and Western Blot analysis.
Results: We found that METD decreases *COL1α1*-mRNA, α-SMA, and TIMP1 protein expression in LX2 cells treated with and TGF-β. This treatment also decreases MFN2 and TIMP1 protein expression and induces overexpression of *MMP2*-mRNA.
Conclusions: Our results suggest that a methanolic extract of *Turnera diffusa* is associated with an antifibrotic effect by decreasing profibrotic and mitochondrial markers together with the possible induction of apoptosis through SNAI1 expression in activated HSC cells.

1. Introduction

The liver can be damaged by a variety of acute, chronic, or intermittent acute insults that in turn induce progressive fibrosis, demonstrated as inflammatory damage, extracellular matrix (ECM) deposition, death of parenchymal cells, and angiogenesis [1]. During liver damage, the liver parenchyma undergoes necrosis and/or apoptosis; the release of cellular contents and reactive oxygen species (ROS) activate hepatic stellate cells (HSCs) and macrophages. HSCs secrete several soluble factors, including cytokines, chemokines, and growth factors, including transforming growth factor-β (TGF-β), which is the main profibrogenic cytokine and stimulates transdifferentiation of HSC to myofibroblasts. Acti-

Abbreviations

TGF-beta	transforming growth factor-beta
HSC	hepatic stellate cells
ECM	extra cellular matrix
RT-qPCR	reverse transcriptase – quantitative polymerase chain reaction
METD	methanolic extract of turnera diffusa

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vated macrophages release platelet-derived growth factor (PDGF) that stimulates the proliferation of myofibroblasts, and these continue to produce TGF-β, perpetuating transdifferentiation, the production of α-smooth muscle actin (α-SMA), collagen type I (COL1α1) matrix metalloproteinases (MMPs), and tissue inhibitor of metalloproteinase (TIMPs), resulting in progressive deposition of ECM and accumulation of tissue damage [2].

In a fibrotic process, changes in the ECM are driven by modulation of MMPs and TIMPs expression. The three most important MMPs expressed in liver injury and fibrosis are MMP2, MMP9, and MMP3 [3]. TIMPs control several functions, which overlap but have different inhibitory profiles for MMPs. TIMP-1 inhibits the active forms of MMP2, MMP7, and MMP9, and its overexpression has been associated with progression to cancer [4].

TGF-β has other pleiotropic functions. It induces the activation of snail family transcriptional repressor 1 (SNAI1), a transcription factor that regulates subsequent processes such as apoptosis and epithelial-mesenchymal transition (EMT) [5].

Also, several chronic liver diseases are associated with early initiating events of mitochondrial damage and dysfunction [6]. Mitochondria are a highly dynamic organelle in constant fission and fusion, and the balance of processes regulates its morphology and normal function [7]. In mammals, the fusion of mitochondria is regulated by two mitofusins, MFN1 and MFN2. In addition to the fusion role, MFN2 participates in mitochondria-mitochondria interaction and the juxtaposition of mitochondria with other organelles, particularly with the endoplasmic reticulum (ER), and its expression has been related to chronic liver disease [8,9].

Currently, some agents stimulate and favor different processes involved in liver regeneration with the consequential reversal of fibrosis. Antifibrotic agents that stimulate liver regeneration more efficiently are still being sought. Since ancient times, plants have been an important source of bioactive compounds against various diseases [10]. Traditional herbal medicine and the use of natural compounds to treat diseases are widely used in society. One of our approaches is the systematic search for natural compounds that show scientific evidence of activity or hepatoprotective properties *in vitro*. We have evaluated several plants from northeast Mexico to determine their usefulness for liver disease treatment [11]. One of these endemic plants, *Turnera diffusa* (TD) has been shown to possess antioxidant and hepatoprotective activity *in vitro*. We demonstrated that HepG2 cells treated with CCl_4 and further exposed to TD extracts showed decreased levels of AST [11]. Furthermore, we isolated a compound, named as hepatodamianol (flavonoid C-glycosylated derived from luteolin) being the main compound responsible for this activity, that showed a maximum enzyme decrease effect in exposed cells [12,13].

Based on this, we aimed to study the potential mechanism(s) by which treatment with a methanolic extract of *Turnera diffusa* (METD) decreases liver injury enzymatic markers in HEPG2 cells, by using an *in vitro* model of a human hepatic stellate (HSC) cell line, LX-2, exposed to METD and evaluating modulation of fibrosis, EMT, and mitochondrial markers.

2. Material and methods

2.1. Preparation of a methanolic extract of Turnera diffusa (METD)

All solvents used in this study were analytical grade, except HPLC-grade methanol (Fisher Scientific, Fair Lawn, NJ). To obtain a flavonoid-rich subfraction we worked with an extract of the aerial part of TD (stems and leaves); subsequently, chlorophylls were removed by solid-phase extraction (phase C-18 cartridge, 1000 mg/8 mL; Alltech). The extracted product was eluted subsequently with aqueous methanol 50%, 70%, and finally, 100% methanol. The fraction obtained with 50% aqueous methanol was fractionated using silica vacuum column chromatography (Silica 60G, Merk Millipore); it was then eluted with a mix of methylene chloride, ethyl acetate, ethyl acetate:methanol 1:1 and methanol. The subfraction obtained with ethyl acetate:methanol 1:1 was the one tested in this work. It was characterized by quantification of hepatodamianol (flavonoid C-glycoside) in a Waters Alliance 1525 liquid chromatography system (Waters, USA) equipped with an online degasser, a binary pump, autosampler, and a 2996 diode array detector (HPLC-DAD) (Waters, USA). Separation was carried out in an inverse phase HypersilGold column (150 mm × 4.6 mm, 5 μm, Thermo Fisher) under the conditions previously established [12].

2.2. Cell culture

We used an *in vitro* model of human hepatic stellate cells (LX-2) kindly donated by Dr. María Luz Martínez Chantar (Metabolomics laboratory, CIC bioGUNE, Spain). Cells were cultured in Dulbecco's Modified Eagle medium (DMEM; Gibco, Grand Island, NY, USA), supplemented with 2% fetal bovine serum (FBS; Gibco, Grand Island, NY, USA), 2 mM L-glutamine, 100 U/mL penicillin, and 100 ug/mL streptomycin, and incubated at 37 °C with a humid atmosphere containing 5% CO_2.

2.3. Cell viability assay

LX-2 cells (4×10^3 cells/well) were seeded in 96-well plates in growth medium containing serum, and after 24 h, cells were treated with 0.1% DMSO (Sigma–Aldrich, Saint Louis, MO, USA) and/or METD (10 to 0.05 mg/mL) for 24, 48, and 72 h. Following incubation, cell viability was evaluated using an MTT [3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay (Roche Diagnostics GmbH Mannheim, Germany), according to the standard experimental protocol. Cell viability was calculated comparing results from METD-treated cells with untreated cells (100% viability).

2.4. HSC exposed to METD treatment

Since METD was dissolved with 0.1% DMSO, we used the same amount of DMSO in all treatments as an untreated control. Human HSC (LX2) were seeded in complete DMEM for 24 h, then cells were treated with a different amount of METD (100 or 200 ng/mL) alone or combined with TGF-β1 (10 ng/mL) (Peprotech, Rocky Hill, NJ) at different times (24, 48, and 72 h) in FBS-free DMEM.

2.5. RNA extraction and Retrotranscription assay

LX-2 cells were seeded at 1×10^5 cells/well in a 24-well plate with complete DMEM for 24 h. The different treatments were added in FBS-free conditions. Cells were harvested at each time point (24, 48, and 72 h) and total RNA was extracted using TRIzol reagent (Invitrogen, Thermo Fisher Scientific, Carlsbad, CA, USA) according to the manufacturer's specifications. RNA was precipitated with 100% isopropanol, washed with 75% alcohol, resuspended in 12 μl RNase-free water, and then stored at −80 °C. Subsequently, 300 ng of RNA was used for cDNA synthesis with the MML-V RT-enzyme (Life Technology; Thermo Fisher Scientific, Carlsbad, CA, USA) as described by the manufacturer.

2.6. Quantitative PCR (qPCR) for fibrosis markers

We used 200 ng of cDNA to perform qPCR to quantify mRNA levels for *α-SMA*, *COL1α1*, *MMP2*, *TIMP1*, *SNAI1*, *MFN2,* and *β-actin*-mRNA as an endogenous control. The real-time PCR thermocycler

used was a StepOne Plus (Applied Biosystems, Foster City, CA, USA). SYBR green was used for detection, and the following primers were used: α-SMA forward (+) 5'-CTACTGCTGAGCGTGAGATTG-3, α-SMA reverse (−) 5-CAGGCAACTCGTAACTCTTCTC; COL1α1 (+) 5-CGATGGATTCCAGTTCGAGTATG-3, COL1α1 (−) 5-CTTGCAGTGGTAGGTGATGTT-3; MMP2 (+) 5-GACAGGTGATCTTGACCAGAAT-3, MMP2 (−) 5-GTGTGTAGCCAATGATCCTGTA-3; TIMP1 (+) 5-CAATTCCGACCTCGTCATCAG-3 and TIMP1 (−) 5-CCTAAGGCTTGGAACCCTTTATAC-3, SNAI1 (+) 5-CCACGAGGTGTGACTAACTATG-3, SNAI1 (−) 5-ACCAAACAGGAGGCTGAAATA-3 and MFN2 (+) 5-CCTTCCTTGAAGACACGTACAG-3, MFN2 (−) 5-GATGCCTCTCACTTTGGATAGG-3. For each qPCR reaction, the following reagents were used: 10 μL of SYBR-Green PCR Master Mix 2x (Applied Biosystems, Foster City, CA, USA), 200–400 nm of each primer, 200 ng of cDNA, completing a total volume of 20 μL. Thermal cycling conditions were 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 60 s. The TaqMan assay was used for β-actin (Applied Biosystems, Foster City, CA, USA), according to the manufacturer's specifications. β-actin expression was used as a housekeeping gene and the fold changes of gene expression were calculated by the $2^{-\Delta\Delta Ct}$ method.

2.7. Protein expression levels of fibrosis markers

LX-2 cells were seeded at 3×10^5 cells/well in a 6-well plate with complete DMEM. The different treatments were added 24 h later in the absence of FBS. Then, total protein extraction was performed at different time points (24, 48, and 72 h), with 1X lysis buffer containing 10 mM Tris-HCl (pH 7.5), 50 mM KCl, 2 mM MgCl2, 1% Triton X-100, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, and Complete Protease Inhibitor Cocktail, according to the manufacture conditions (Roche, Mannheim, Germany), and then quantified by the Bradford method (Bio-Rad, Hercules, CA, USA). We used 50 μg of protein for 12% SDS-PAGE gels and transferred them to PVDF membranes (Amersham Biosciences, Freiburg, Germany). Membranes were incubated with the following antibodies, anti-α-SMA (ab32575, Abcam, Cambridge, MA, USA) at a dilution 1:1000, anti-TIMP1 (ab109125, Abcam) at a dilution 1:1000, MFN2 (Ab56889, Abcam) at a dilution 1:1000 and anti-GAPDH (MAB5718, R&D Systems, Minneapolis, MN, USA) at a dilution of 1:2500. Membranes were washed with TBS-Tween, then incubated with horseradish-peroxidase-conjugated goat anti-mouse IgG or goat anti-rabbit IgG (Promega, Madison, WI, USA) both at a dilution 1:10,000. Signal detection was measured by chemiluminescence using a Clarity™ Western ECL Substrate (Bio-Rad, Hercules, CA, USA) and ChemiDoc Imaging Systems (Bio-Rad).

2.8. Statistical analysis

The data were analyzed using Student's *t*-test or one-way analysis of variance (ANOVA) for comparison between any two groups or among multiple groups, respectively. The program used was GraphPad Prism 6 (Northside Dr. Suite, San Diego, CA, USA). All data were expressed as mean ± SEM and a *p*-value <0.05 was considered a significant difference.

3. Results

3.1. METD treatment is not cytotoxic for LX-2 cells

First, METD was analyzed by high-performance liquid chromatography with diode array detection (HPLC-DAD) for the quantification of hepatodamianol present in the METD. Fig. 1A shows a representation of a profile of METD measured (chromatogram) in milli-absorbance units (mAU) through time (retention time, RT). We identified that at a wavelength of 280 and 350 nm, where a maximum RT can be observed at 8 min, the hepatodamianol estimated concentration was between 5.35 ± 0.12% [12].

Based on this, we then proceeded to determine the METD concentrations that are not cytotoxic for the LX-2 cell line by measuring cytotoxicity using an MTT assay. A range of METD concentrations from 0.05 to 10 mg/mL at 24 h (Fig. 1B) was used to treat cells up to 72 h and choose the concentrations that do not decrease cell viability below 75%. We observed that cell viability was above 60% in concentrations less than 1 mg/mL for 24 h; contrary to that, a significant reduction in cell viability (<20%) was observed when cells were incubated at 5.0 and 10.0 mg/mL (Fig. 1B). We also determined that METD 0.5 mg/mL treatment at 24 h, inhibited proliferation of LX-2 cells in a concentration-dependent manner (Fig. 1B). The inhibitory concentration 50 (IC50) of METD extract was 1.148 mg/mL in LX-2 cells (Fig. 1C). Based on this, the evaluation of the cytotoxicity of the METD was performed using a range of concentrations from 0.05 to 5.0 mg/mL in LX-2 cells at different time points (24, 48, and 72 h). We found that METD 0.15 mg/mL, decreases cell proliferation in a time-dependent manner (Fig. 1D). At concentrations of 1.0 and 5.0 mg/mL METD inhibited proliferation at early time points (24 h), while at concentrations of 200 to 500 ng/mL cell viability decreased to less than 80% at 72 h and concentrations less than 150 ng/mL maintain cell viability around 80% (Fig. 1D). Based on these results, we decided to use the METD at the concentrations of 100 and 200 ng/mL until 72 h because it demonstrated no cytotoxic effect. This was done to further evaluate the effect of the METD in the presence of TGF-β in this cell line.

3.2. METD does not modify sustained LX-2 cell morphology in the presence of TGF-β.

To assess whether exposure to METD induces morphology disruption (Fig. 2), LX-2 cells were cultured in free-serum conditions in the presence of METD (200 ng/mL) alone or combined with TGF-β (10 ng/mL) at three different time points (24, 48 and 72 h) and were evaluated under a phase-contrast microscope. We observed that LX-2 cells in the presence of METD (Fig. 2B, 24 h) preserve a stellate shape, but when the cells were exposed to TGF-β (10 ng/mL), cell morphology changed to an adherent spindle shape (stretching) forming clusters and leaving wide spaces between them (Fig. 2C, 24 h). In addition, when the cells were exposed to combined treatment with TGF-β (10 ng/mL) and METD (200 ng/mL), this pattern was not observed (adherent spindle shape and clusters) and most of the cells kept their stellate shape (Fig. 2D, 24 h). Most dead cells were observed upon treatment with TGF-β (10 ng/mL) at 48 h, and this effect was attenuated by the METD treatment. This pattern is similar to cells treated until 72 h but showing a greater amount of cell death.

3.3. METD attenuates fibrogenic expression markers in LX-2 cells.

We wanted to know whether METD could suppress LX-2 cell activation (Fig. 3). For this purpose, LX-2 cells were treated with METD (100 and 200 ng/mL) alone o combined with TGF-β (10 ng/mL) in FBS-free conditions at three different time points (24, 48, and 72 h). We evaluated the expression of *COL1α1*-mRNA (Fig. 3A) through RT-qPCR in METD-treated cells at different times described above. We found that treatment with METD (100 or 200 ng/mL) decreases *COL1α1*-mRNA levels expression. Only the concentration of 200 ng/mL of METD inhibited *COL1α1*-mRNA expression at all times. We observed that, even at 72 h, it has 65% more inhibition of *COL1α1*-mRNA expression than 100 ng/mL of METD. As expected, treatment with TGF-β (10 ng/mL) increased *COL1α1*-mRNA level expression at all times, and in the presence

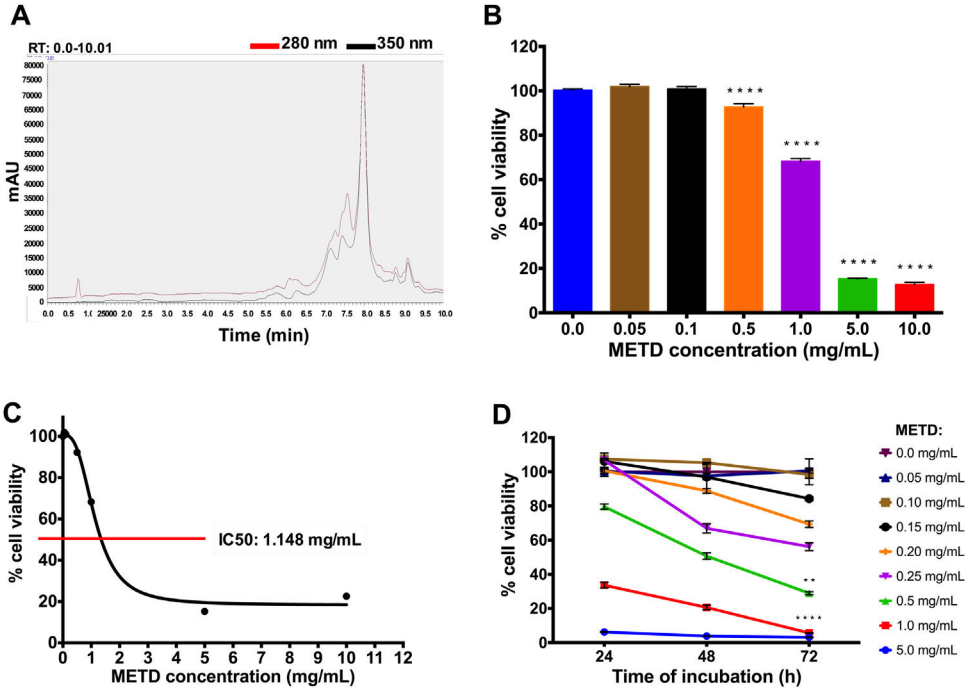

Fig. 1. Effect of METD on the cell viability of LX-2. (A) Chromatographic profile of METD by High-Performance Liquid Chromatography with Diode-Array Detection (HPLC-DAD). (B) Evaluation of cell viability at 24 h. LX-2 cells were treated with the METD (0–10 mg/mL) for 24 h; viability was measured by the MTT assay, the bars indicate the percentage of cell viability. (C) IC50 of METD. D) Evaluation of cell viability at concentrations of 5.0 to 0.05 mg/mL of METD for 24, 48, and 72 h in LX-2 cells. All experiments were performed in triplicate. $^{*}p > 0.05$, $^{**}p > 0.01$, $^{***}p > 0.001$ and $^{****}p > 0.0001$.

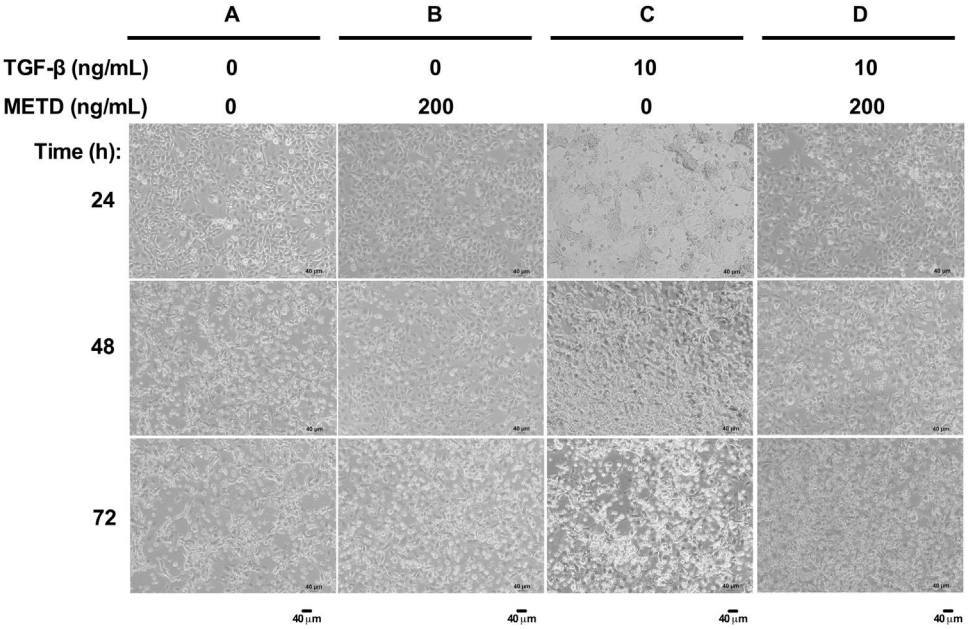

Fig. 2. Effect of METD on the morphology of LX-2 cells treated with TGF-β. LX-2 cells were treated with 200 ng/mL of METD alone or combined with TGF-β (10 ng/mL) for 24, 48, and 72 h and then images were observed under a phase contrast microscope.

of METD, this effect was attenuated in both concentrations used (100 or 200 ng/mL), but the concentration of 200 ng/mL of METD presented the maximum effect in the inhibition of *COL1α1*-mRNA levels expression *versus* TGF-β (10 ng/mL) (Fig. 3A), inhibiting expression at all times and being maintained until 72 h, reaching the levels of basal *COL1α1*-mRNA expression from 48 h to 72 h (Fig. 3A).

Furthermore, we evaluated transcriptional and translational α-SMA level expression (Fig. 3A and B, respectively). In the transcriptional evaluation, the presence of METD (100 or 200 ng/mL) *α-SMA*-mRNA levels were increased and perpetuating up to 72 h. Treatment with TGF-β (10 ng/mL) increases *α-SMA*-mRNA level expression at 72 h, and the presence of METD (100 or 200 ng/mL) enhances this effect. METD (100 ng/mL) decreases α-SMA protein expression at all times but at a concentration of 200 ng/mL, this effect was observed only upon 48 h (Fig. 3B). In the presence of TGF-β (10 ng/mL) and METD, there was α-SMA protein downreg-

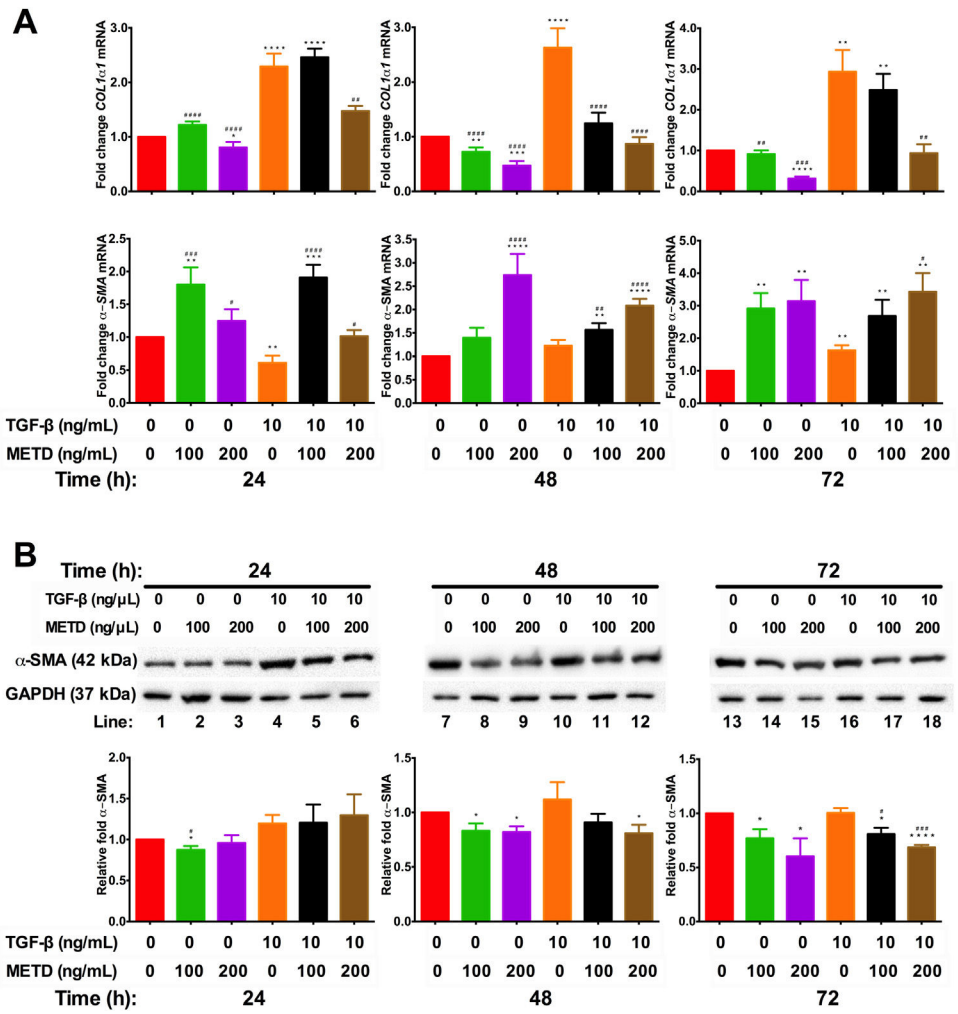

Fig. 3. Effect of METD on pro-fibrotic markers in LX-2 cells treated with TGF-β. (A) *COL1α1* and *α-SMA* mRNAs were measured by real-time PCR. LX2 cells were plated in 24-well plates, incubated in serum-free medium for 24 h, followed by METD treatment (100 or 200 ng/mL) at 24, 48, and 72 h, alone or combined with TGF-β (10 ng/mL). *β-actin* mRNA expression was used as control. B) α-SMA protein expression was analyzed by Western blot. LX2 cells were plated in 6-well plates and treated as above. GAPDH protein was used as a control. All experiments were performed in triplicate. *Condition *vs.* SFB-free and #condition *vs.* TGF-β alone (*$p < 0.005$, **$p < 0.01$, ***$p < 0.001$ and ****$p < 0.0001$).

ulation from 48 h at 200 ng/mL of METD and was only observed at 72 h for 100 ng/mL of METD.

3.4. *METD modulates TIMP1 expression*

Since we observed that METD lowers expression levels of *COL1α1*-mRNA and α-SMA protein, we wanted to assess whether METD was modifying extracellular matrix modulators such as MMP2 and TIMP1 (Fig. 4). Therefore, LX-2 cells were treated with METD (100 and 200 ng/mL) alone o combined with TGF-β (10 ng/mL) in FBS-free conditions at three different time points (24, 48, and 72 h).

We observed that *MMP2*-mRNA expression (Fig. 4A) was increased in cells in the presence of METD (100 and 200 ng/mL) and when cells were further exposed to TGF-β (10 ng/mL), this effect was enhanced. There was *TIMP1*-mRNA overexpression (Fig. 4A) in LX2 cells in presence of METD (100 and 200 ng/mL) and the pattern repeated when the cells were also exposed to TGF-β (10 ng/mL), showing enhanced overexpression levels. But TIMP1 protein expression (Fig. 4B) showed downregulation in LX2 cells in the presence of METD (48 and 72 h), with 30% for 100 ng/mL of METD to 44% for 200 ng/mL of METD with greater inhibition at 72 h; in combined treatment of TGF-β (10 ng/mL) and METD, there was a decrease in TIMP1 protein expression levels at 72 h (100 ng/mL of METD) and at all times (200 ng/mL of METD).

3.5. *METD increases* SNAI1*-mRNA expression*

To assess whether METD participates in the EMT mechanism (Fig. 5), LX-2 cells were treated with METD (100 and 200 ng/mL) alone or combined with TGF-β (10 ng/mL) in FBS-free conditions at three different time points (24, 48, and 72 h) and then *SNAI1*-mRNA expression was evaluated. In the presence of METD, there was *SNAI1*-mRNA overexpression at all times, which increased even more than with stimulation with TGF-β (10 ng/mL), and with the stimulation of both (METD and TGF-β), expression was up to 4 times more than the control.

3.6. *METD modulates MFN2 expression*

To assess mitochondrial function in the presence of METD, LX-2 cells were treated with METD (100 and 200 ng/mL) alone or combined with TGF-β (10 ng/mL) in FBS-free conditions at three different time points (24, 48, and 72 h). We evaluated MFN2 mRNA and protein expression levels (Fig. 6) and found that *MFN2*-mRNA levels were upregulated in the presence of METD at all times, even

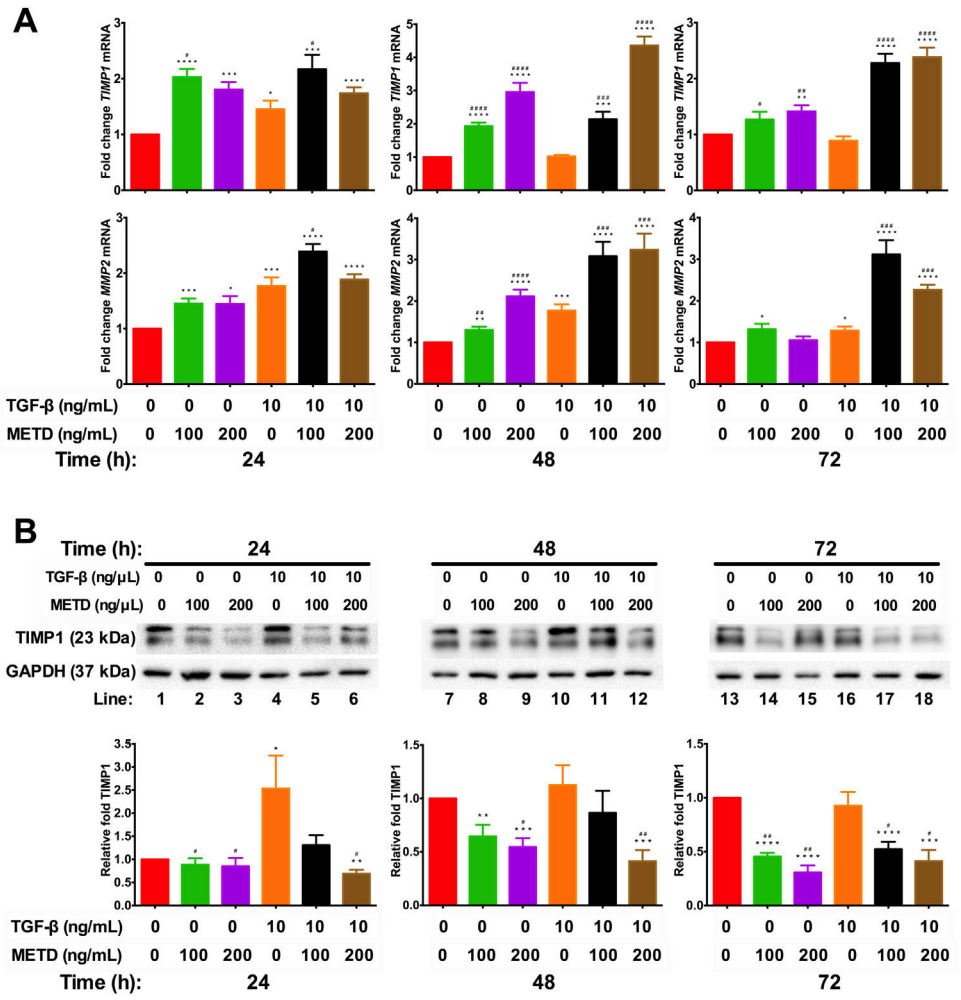

Fig. 4. Effect of METD on extracellular matrix modifiers in LX-2 cells treated with TGF-β. (A) *TIMP1* and *MMP2* mRNAs were measured by real-time PCR. LX2 cells were plated in 24-well plates, incubated in serum-free medium for 24 h, followed by METD treatment (100 or 200 ng/mL) upon 24, 48, and 72 h, alone or combined with TGF-β (10 ng/mL). *β-actin* mRNA expression was used as control. (B) TIMP1 protein expression was analyzed by Western blot. LX2 cells were plated in 6-well plates and treated as above. GAPDH protein was used as a control. All experiments were performed in triplicate. *Condition *vs.* SFB-free and #condition *vs.* TGF-β alone (*$p < 0.005$, **$p < 0.01$, ***$p < 0.001$ and ****$p < 0.0001$).

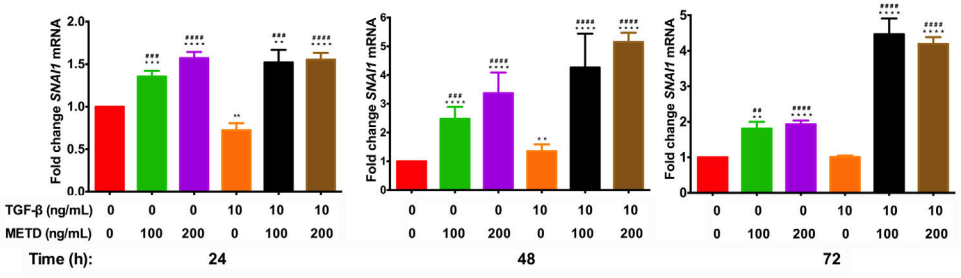

Fig. 5. Effect of METD on *SNAI1* mRNA expression in LX-2 cells treated with TGF-β. *SNAI1* mRNA expression was measured by real-time PCR. LX2 cells were plated in 24-well plates, incubated in serum-free medium for 24 h, followed by METD treatment (100 or 200 ng/mL) upon 24, 48, and 72 h, alone or combined with TGF-β (10 ng/mL). *β-actin* mRNA expression was used as a control. All experiments were performed in triplicate. *Condition *vs.* SFB-free and #condition *vs.* TGF-β alone (*$p < 0.005$, **$p < 0.01$, ***$p < 0.001$ and ****$p < 0.0001$).

more than upon stimulation with TGF-β. The stimulation with both (METD and TGF-β) also caused overexpression at all times and this effect was more evident in the dose of 200 ng/mL of METD to 72 h (Fig. 6A). MFN2 protein expression was decreased in the presence of METD at 24 (only for 200 ng/mL) and 48 h (both doses); in the presence of METD and TGF-β, it decreased compared to TGF-β at all times, even compared to control at 48 and 72 h (Fig. 6B).

4. Discussion

Hepatic stellate cells are a major cell type responsible for liver fibrosis following their activation into fibrogenic myofibroblast-like cells [14]. We explored the potential of METD as an antifibrotic agent and its role in EMT modulation and mitochondria dynamics in LX-2 cells. Liver fibrosis is in part the result of the imbalance between synthesis and degradation of ECM proteins. Activated

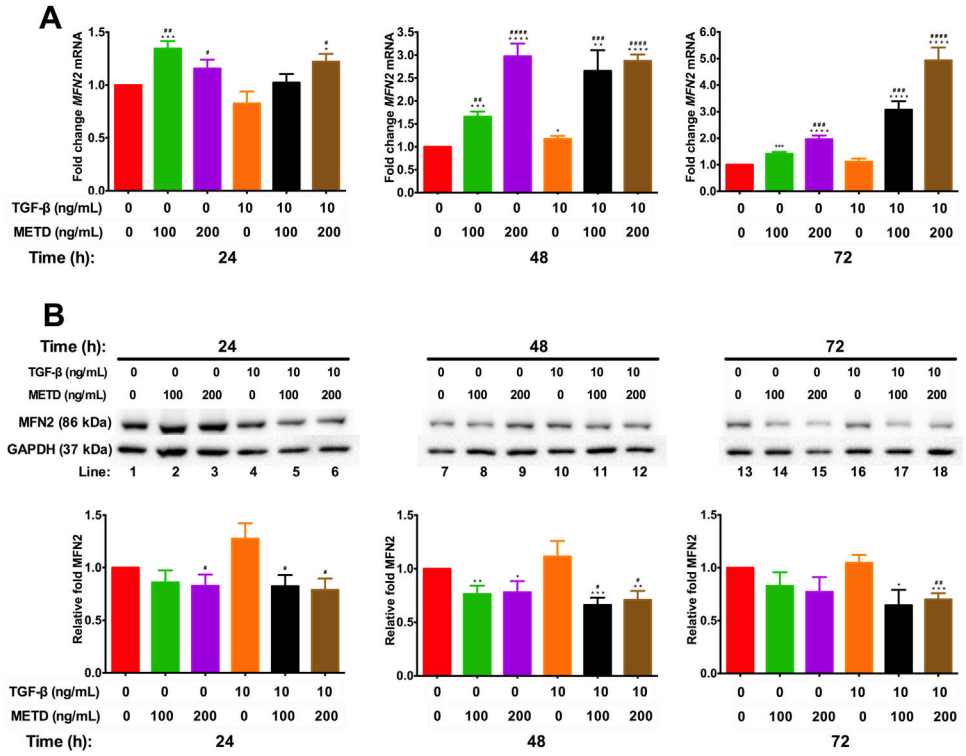

Fig. 6. Effect of METD on MFN2 mRNA and protein expression in LX-2 cells treated with TGF-β. (A) *MFN2* mRNAs were measured by real-time PCR. LX2 cells were plated in 24-well plates, incubated in serum-free medium for 24 h, followed by METD treatment (100 or 200 ng/mL) upon 24, 48, and 72 h, alone or combined with TGF-β (10 ng/mL). *β-actin* mRNA expression was used as a control. (B) MFN2 protein expression was analyzed by Western blot. LX2 cells were plated in 6-well plates and treated as above. GAPDH was used as a control. All experiments were performed in triplicate. *Condition *vs.* SFB-free and #condition *vs.* TGF-β alone ($^{*}p < 0.005$, $^{**}p < 0.01$, $^{***}p < 0.001$ and $^{****}p < 0.0001$).

HSCs express a combination of MMPs and TIMPs in different stages of the disease through the TGF-β pathway. They specifically express high levels of TIMP1 and MMP2. TIMP1 inhibits MMPs activity, perpetuating the accumulation of ECM whereas MMP2 activity is inhibited by TIMP1 [15,16].

COL1α1 is a marker used for fibrosis and METD causes a significant decrease in *COL1α1*-mRNA expression, 69% less than the basal expression of HSC in FBS-free conditions and the inhibition was dose-dependent. METD decreased *COL1α1*-mRNA expression in the presence of TGF-β also in a dose-dependent manner, even reaching the level of basal expression of HSC in FBS-free conditions, indicating that METD is a potent inhibitor of *COL1α1*-mRNA expression in a dose-dependent manner. In the fibrotic process, when the ECM is modified, in part by the accumulation of COL1α1 protein and cross-linking [17], it creates a pro-fibrotic environment that can activate MMP2 [18] and this, in turn, can activate TGF-β latent, and/or unlink the ECM integrins promoting apoptosis [19].

During HSC activation there is upregulation of smooth muscle-specific genes (among them α-SMA) and actin polymerization is increased. This promotes the release of ECM molecules like MRTF/A (actin-binding protein) and TGF-β activation that further increase the fibrotic response by stimulating the expression of genes such as *α-SMA* and *COL1α1* [20]. The METD increased *α-SMA*-mRNA expression and decreased α-SMA protein levels, in the presence or absence of TGF-β. This decrease of the α-SMA protein modulated by METD will diminish fibrotic stimulation and transmission of mechanical signals through actin polymerization, reducing the activation of TGF-β, release binding proteins to actin and therefore, lowering the fibrotic response by a decrease in α-SMA and COL1α1 expression.

We observed that LX-2 cells exposed to METD treatment alone or combined with TGF-β showed *TIMP1*-mRNA levels were upregulated compared with cells in FBS-free conditions but the METD in both concentrations used alone or combined with TGF-β decreased the levels of TIMP1 protein expression. Numerous studies suggest a positive association of TIMP1 with hepatic fibrogenesis and with progression to cancer [4]. TIMP1 inhibition is associated with a favorable fibrosis resolution but it has also been shown that the cell type and the microenvironment (a molecular network of factors) play a very important role; for example, the rigidity of the ECM [17] and the presence of other molecules [21,22].

According to the aforementioned, we expect that a decrease in TIMP1 protein expression could be modulated by METD and could have a favorable fibrotic resolution; however, the decrease or irruption of a stimulus would not be sufficient for the decrease or regression of a complex process, such as fibrosis [23].

MMPs are central to the fibrosis remodeling process and MMP activity is controlled at various levels: modulation of gene expression, compartmentalization, availability of activators, and inhibition [24,25]. METD alone or combined with TGF-β induced an increase in *MMP2*-mRNA expression with respect to cells in FBS-free conditions. MMP2 has a dose-dependent activation for COL1α1[24], if METD decreases the COL1α1 protein level, MMP2 activation could be affected. Also, MMP2 can activate latent TGF-β [26]; therefore, METD will decrease ECM deposition (a decrease in α-SMA and COL1α1 protein), and MMP2 and TGF-β activation. The induction of *MMP2*-mRNA overexpression by METD is not indicative of translational overexpression, so we do not know if METD is causing MMP2-mediated apoptosis [19].

SNAI1 is a downstream target of TGF-β and plays a key role in regulating several following cell fate decisions such as apoptosis and EMT induction (relates fibrogenesis to carcinogenesis) [5]. *SNAI1*-mRNA expression is different in distinct cell lines [27,28]. In this work, only at 48 h, we found that *SNAI1*-mRNA levels were

upregulated with the stimulation of TGF-β in relation to cells in FBS-free conditions. METD alone or combined with TGF-β increases *SNAI1*-mRNA expression. We hypothesized that *SNAI1*-mRNA overexpression is not reflected at the translational level or that there is some translational regulation that does not allow its activity because SNAI1 represses *α-SMA*-mRNA expression [29] and this does not happen, since, despite *SNAI1*-mRNA overexpression caused by the METD, there is *α-SMA*-mRNA overexpression at all times evaluated.

Mitochondria participate in almost all aspects of cell function and have been linked to pathogenic processes. Specifically, MFN2 expression has been related to chronic liver disease [8,9]. METD alone or combined with TGF-β causes *MFN2*-mRNA overexpression and decreased MFN2 protein expression. The modulation caused by METD and the probable cell triggering is not clear because MFN2 plays a role in the balance between apoptosis and autophagy and lack evaluation of the response caused in the cell context.

In this work, there were discrepancies in mRNA and protein expression in different genes (*α-SMA*, *TIMP1*, and *MFN2*) in the presence of METD alone or combined with TGF-β, indicating that METD is causing differential regulation of expression between mRNA and protein; thus, we hypothesize that transcriptional and translational regulatory mechanisms are being activated differently. These mechanisms of transcriptional regulation can be mediated by trans-acting factors, RNA-binding proteins, microRNA, and lncRNA that interact with cis-elements located in the mRNA, with activity profibrotic or anti-fibrotic [30]. While translational regulation through protein degradation (ubiquitination) [30,31] could influence the discrepancy of mRNA and protein expression. Previous work [32] reported that miR-130 can regulate genes related to activation of HSC in a rat model, while miR-205 moderates the TGF-β/Smad signaling effect by direct downregulation of Smad [33].

We found evidence that METD in the presence of TGF-β decreases *COL1α1*-mRNA, α-SMA, and TIMP1 protein expression. It also decreases MFN2 and TIMP1 protein expression and overexpression of *MMP2*-mRNA. We suggested a probable mechanism by which METD could perform its hepatoprotective role is by inducing apoptosis of activated HSC cells. This effect must be verified in animal models, and the routes by which METD is modulating these markers should be explored. The increase in *SNAI1*-mRNA expression caused by METD was not explored in this work. Plant extracts, specifically flavonoids, are potential compounds in the search for antifibrotic drugs [34].

The fibrotic and HSC activation mechanism toward myofibroblast differentiation is a complicated molecular process in which multiple factors are involved; therefore, the mechanism for fibrogenesis reduction *in vivo* is complex and multifactorial. Cell type, the sequence of events, cell-cell interaction, and cell-ECM interaction intervene in the fibrotic process, in addition to the regulation triggered directly by a stimulus; therefore, the true activity of the molecules produced by the triggering of the stimulus will be subject to transcriptional, translational, functional and spatial regulation [17,30,31,35]. Further evidence should be generated to identify the related mechanisms involved in the hepatoprotective role of natural compounds.

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Conflict of interest

The authors have no conflicts of interest to declare.

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