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Methoxyeugenol deactivates hepatic stellate cells and attenuates liver fibrosis and inflammation through a PPAR-ɣ and NF-kB mechanism

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ABSTRACT

Ethnopharmacological relevance: Studies have shown interest in nutraceuticals for the prevention of liver diseases. Methoxyeugenol, is a molecule found in foods, such as nutmeg (*Myristica fragrans Houtt.*) and Brazilian red propolis. These two sources of methoxyeugenol, propolis and nutmeg, are used in folk medicine for the treatment of hepatic and gastrointestinal disorders, although little is known about their effects on the prevention of liver fibrosis. Natural PPAR (Peroxisome proliferator-activated receptor) agonists would represent unique molecules for therapy, considering the lack of therapeutics to treat liver fibrosis in chronic liver disease. Thus, investigation on new alternatives are necessary, including the search for natural compounds from renewable and sustainable sources. Liver fibrosis is a pathological process characterized by an exacerbated cicatricial response in the hepatic tissue, which compromises liver function. Therefore, inhibition of HSC (hepatic stellate cell) activation and hepatocyte damage are considered major strategies for the development of new anti-fibrotic treatments.

Aim of the study: This study aimed to investigate the effects of methoxyeugenol treatment on HSC phenotype modulation in human and murine cells, hepatocyte damage prevention, and protective effects *in vivo*, in order to evaluate its therapeutic potential for liver fibrosis prevention.

Methods: We investigated the effects of methoxyeugenol in (i) *in vitro* models using human and murine HSC and hepatocytes, and (ii) *in vivo* models of CCl_4 (carbon tetrachloride) -induced liver fibrosis in mice.

Results: We herein report that methoxyeugenol decreases HSC activation through the activation of PPAR-ɣ, ultimately inducing a quiescent phenotype highlighted by an increase in lipid droplets, loss of contraction ability, and a decrease in the proliferative rate and mRNA expression of fibroblast markers. In addition, methoxyeugenol prevented hepatocytes from oxidative stress damage. Moreover, in mice submitted to chronic liver disease through CCl_4 administration, methoxyeugenol decreased the inflammatory profile, liver fibrosis, mRNA expression of fibrotic genes, and the inflammatory pathway signaled by NF-kB (Nuclear factor kappa B).

Conclusion: We propose methoxyeugenol as a novel and potential therapeutic approach to treat chronic liver disease and fibrosis.

1. Introduction

Natural compounds, crude extracts, or isolated molecules obtained from plants have progressively attracted attention for devising antifibrotic therapies (Bravo, 2009; Singh et al., 2005). Methoxyeugenol (4-Allyl-2,6-dimethoxyphenol) is a molecule present in the human diet, as it is widely used as an additive in the food industry, and it is also present in the composition of several plant that showed beneficial effects for treatment of innumerous diseases (Agnihotri et al., 2012; Righi et al.,

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Abbreviations list

HSC	Hepatic stellate cell
TGF-β:	Transforming growth factor beta
PPAR	Peroxisome proliferator-activated receptor
NF-kB	Nuclear factor kappa B
DMEM	Dulbecco's modified eagle medium
CCl_4	Carbon tetrachloride
FBS	Fetal bovine serum
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium
DMSO	Dimethyl sulfoxide
NAC	N-acetylcysteine
DPPH	2,2-Diphenyl-1-picrylhydrazyl
TBARS	Thiobarbituric acid reactive substances
LDH	Lactate dehydrogenase
SLM	Silymarin
ALT	Alanine transaminase
AST	Aspartate transaminase
NAS	Nonalcoholic Fatty Liver Disease Activity Score
PBS	Phosphate Buffered Saline
DAB	3,3′-Diaminobenzidine
α-SMA	Alpha-smooth muscle actin
p-NF-kB	Phosphorylated nuclear factor kappa B
Col1a1	Type I collagen
PDGFrβ	Platelet-derived growth factor receptor beta
IL:	Interleukin
TNF-α:	Tumor necrosis factor alpha
CD163	Cluster of differentiation 163
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
TTBS	Tween-tris-buffered saline
BSA	Bovine serum albumin
EDTA	Ethylenediamine tetraacetic acid
iNOS	Inducible NO synthase
LPS	Lipopolysaccharide

2011). It is found in herbs, spices, sassafras oil (*Sassafras albidum*), nutmeg (*Myristica fragrans Houtt.*), *Cinnamomum glanduiferum*, and Brazilian red propolis (Agnihotri et al., 2012; Guridip Singh et al., 2005; Kamdem and Gage, 1995; Righi et al., 2011).

As mentioned, Methoxyeugenol has been identified in Brazilian red propolis, which is produced by bees of the species *Apis mellifera,* collected from plants exudate of the *Leguminosae* family (*Dalbergia ecastaphyllum (L.) Taub)* found in Maceió, in the northeast of Brazil (Boeing et al., 2020; Righi et al., 2011). Propolis therapeutic effects are known for centuries in folk medicine and used to treat infections, gastric disorders and also to improve wound healing (Sforcin, 2016; Ghisalberti, 1979). Moreover, the therapeutic effect of red propolis has been investigated for treatment of hepatic and gastric disorder and showed hepatoprotective and gastroprotective properties in mice (Boeing et al., 2020; Silva et al., 2019).

Nutmeg (*Myristica fragrans Houtt.*), another important source of methoxyeugenol, is a seed used as spice, originally from Indonesians islands, that was widely spread to the world by the English exploration in the 17th century (Abourashed and El-Alfy, 2016). Its use in folk medicine is reported mainly in the treatment of gastrointestinal problems and has long been used as a therapy for this kind of illness (Asgarpanah and Kazemivash, 2012). Moreover, studies showed that nutmeg could be useful in the treatment of nonalcoholic fatty liver disease (Zhao et al., 2020), promoting lipid metabolism regulation and anti-inflammatory effects. More importantly, extract containing nutmeg could effectively protect liver against hepatic toxic-induced damage (Yimam et al., 2016). Nevertheless, the effects of methoxyeugenol on liver fibrogenesis and the associated molecular mechanisms of its possible beneficial effects are still unknown.

Liver fibrosis has its development linked to chronic liver damage, often due to viral infections, alcoholism, and toxins (Friedman, 2008). Chronic injury leads to an inflammatory process that ultimately results in HSCs activation, with the acquisition of a myofibroblastic phenotype, and the initiation of the fibrotic process, evidenced by the loss of synthesis/degradation balance of extracellular matrix elements (Iredale, 2008). The maintenance of HSCs phenotype is primarily performed by transcription factors, which tightly modulate the function of these cells. In a quiescent state, HSCs act on glucose regulation, lipid synthesis and degradation and synthesis of extracellular matrix components (Guimarães et al., 2007; Marrone et al., 2016). Nevertheless, upon activation, HSCs become pro-contractile, pro-inflammatory, and pro-fibrogenic (Gracia-Sancho et al., 2018), actively contributing to chronic liver disease progression.

Among the transcription factors regulating HSCs, the PPAR family (mainly PPAR-ɣ and PPAR-α) has demonstrated to play an important role in promoting their quiescent phenotype (Ivanova et al., 2015; Tyagi et al., 2011; Chen et al., 2015) and inhibiting the activation of inflammatory pathways, manly those activated by NF-kB signaling (Mirza et al., 2019; Qu et al., 2017). Therefore, modulators of these transcription factors are promising candidates to initiate the process of HSCs deactivation.

It is nowadays clear that hepatic fibrosis treatment requires the identification and removal of the chronic damage agent and/or the administration of drugs with beneficial activities on proliferation and activation of the hepatic stellate cells, oxidative stress, and inflammation. Currently, treatments for liver fibrosis includes curcumin, quercetin, silymarin and probiotics that reduce oxidative stress and inflammation in the liver. Furthermore, more recently, the potential for treatment with small interfering RNAs to decrease TGF-β signaling has been demonstrated to be a promising strategy. (Pragyan et al., 2021; Popov and Schuppan, 2009; Tighe et al., 2020; Liedtke et al., 2013). On the other hand, natural PPAR agonists would represent unique molecules for therapy, as they exert beneficial effects with fewer side effects than specific agonists of these receptors (Wang et al., 2014).

In a previous work by our research group, methoxyeugenol showed an effect in decreasing cell proliferation in human endometrial cancer (Costa et al., 2021). HSCs are responsible for extracellular matrix deposition and synthesis, and their activation and proliferation rate are closely linked to the development of fibrosis. Considering the lack of therapeutics to treat liver fibrosis in chronic liver disease, investigation on new alternatives are necessary, including the search for natural compounds from renewable and sustainable sources (Bravo, 2009). In the present study, we investigated the effects of methoxyeugenol on HSCs phenotype in human and murine cells, hepatocyte damage prevention, and protective effects *in vivo*, in order to evaluate its therapeutic potential for liver fibrosis prevention.

2. Materials and methods

2.1. In vitro experiments

2.1.1. Cell culture

The murine HSC cell line GRX was obtained from the Rio de Janeiro Cell Bank (HUCFF, UFRJ, Rio de Janeiro, Brazil) and the LX-2 was kindly provided by Dr. Bataller. Cells were maintained in DMEM (Dulbecco's Modified Eagle's Medium, Sigma, 5030) supplemented with 10% FBS (fetal bovine serum, Gibco, 26140079), 1% penicillin and streptomycin (Gibco, 15140122) and pH 7.4. Cells were seeded in tissue culture plates and, after 24 h, treated and incubated for 72 h at 37 °C in a humidified atmosphere of 5% CO_2. HepG2 (obtained from the Rio de Janeiro Cell

Bank - HUCFF, UFRJ, Rio de Janeiro, Brazil) and VERO cells (kindly provided by Dr. Pablo Machado) were maintained in DMEM supplemented with 10% FBS and subjected to the same conditions and temperature mentioned above. All *in vitro* experiments were performed four independent times, in triplicate.

Methoxyeugenol molecule (4-Allyl-2,6-dimethoxyphenol) was purchased from Sigma-Aldrich (Cat.#W365505/Lot#STBD5682V) and stored in room temperature. For experiments, methoxyeugenol was solubilized in DMSO (dimethyl sulfoxide).

2.1.2. Cellular viability

Cellular viability of GRX, VERO and HepG2 cells were determined by colorimetric MTT (2,2-Diphenyl-1-picrylhydrazyl, Sigma, 298-93-1) assay (De Mesquita et al., 2013). Briefly, 3×10^3 cells per well were seeded in 24-well plate, grown for 24 h and treated with methoxyeugenol at 15, 30, 60, 125 and 250 μM diluted in 0.5% DMSO (Synth, 01D1011) for 72 h. After that, MTT solution was added and cells were incubated for 4 h. NAC (N-acetylcysteine) was used as positive control at 2.5 mM. Formazam crystals were dissolved with DMSO and quantified in an ELISA microplate reader at absorbance of 492 nm. For cell number determination, cells were counted in Neubauer chamber with Trypan blue Solution 0.4% (Gibco, 15250061) and live cells expressed as control percentage. Considering that 30 μM was the lowest concentration that induced a decrease in cell proliferation and did not show cellular toxicity, this dose was chosen for further experiments.

2.1.3. Antioxidant activity

Methoxyeugenol was evaluated for its antioxidant activity by DPPH (2,2-diphenyl-1-picrylhydrazyl, Sigma, 1898-66-4) reduction (Dias et al., 2017). The antioxidant activity was measured by spectrophotometry in an ELISA reader at the wavelength of 515 nm. All samples analyzed were dissolved in methanol 100%. Ascorbic acid (550 μg/mL) was used as positive control due to its well-known antioxidant activity.

2.1.4. Oxidative stress in HepG2 cells

For oxidative stress evaluation, HepG2 cells were seeded in 6-well plate at concentration of 1×10^5 cells per well. After 6 h, cells were adhered to the well bottom and received the pre-treatment of methoxyeugenol at 30 μM for 12 h. Later, cells were challenged with CCl_4 (4 mM) for 6 h. At the end of the experiment, both supernatant and cell lysate were collected. The concentration of TBARS (thiobarbituric acid reactive substances) and LDH (lactate dehydrogenase, Labtest, 86) released were analyzed (Krause et al., 2017).

2.1.5. Detection of lipid droplets in HSC

Lipid droplets accumulation in HSC was observed using Oil Red staining (De Mesquita et al., 2013). Cells were plated in a 24-well tissue culture plate (3×10^3 cells/well), and 72 h after treatment with methoxyeugenol, cells were fixed with 10% formaldehyde and stained with Oil Red-O (Sigma, 1320-06-5). Intracellular lipid accumulation was observed after 30 min, using an inverted light microscope at magnification of 400×. The dye from stained cells was extracted using isopropanol and specific lipid content was calculated as the ratio of absorbance value and number of cells. Oil Red-O quantification was accessed by optic density at 492 nm.

2.1.6. Assessment of cell contraction by collagen gel assay

Collagen gels consist of a solution with collagen 4 mg/mL and DMEM 4× concentrated. Each gel was impregnated with 1×10^5 cells and added to a 24-well plate, left to polymerize for 30 min at 37 °C, detached and suspended in 600 μL of DMEM with 5% FBS solely or with either methoxyeugenol or NAC. Images were obtained 24 h after and surface area of each gel was determined as a percentage of the total well area occupied (De Oliveira et al., 2020).

2.1.7. PPAR- ɣ antagonist pre-treatment

Human activated HSC LX-2 were seeded in a 6-well plate at concentration of 4×10^4 cells per well and pre-treated with specific PPAR-ɣ antagonist GW9662 (Sigma, M6191-5 MG), at concentration of 10 μM for 24 h (Gionfriddo et al., 2020). Later, cellular medium was changed to a new medium contain DMSO, for the control and GW992 groups, and DMSO + methoxyeugenol 30 μM for the treated group. Cells were maintained in incubation for an additional of 72 h and mRNA were collected to evaluate the activation HSC markers.

2.2. In vivo experiments

2.2.1. Animals and experimental design

Male BALB/c mice, 8 weeks old and weighing 25–30 g, were bred and maintained at the university animal facilities (CeMBE, PUCRS), under specific pathogen free conditions, 12/12 h light–dark cycle, temperature of 22 °C with free access to water and food on individually ventilated cages.

Liver fibrosis was induced using the chronic CCl_4 administration model. Forty BALB/c mice were randomly allocated to one of the following 4 groups (ten mice per group): Control, CCl_4, CCl_4 + methoxyeugenol 0.25 mg/kg and CCl_4 + methoxyeugenol 1.0 mg/kg. Mice were i.p. (intraperitoneally) injected with CCl_4 (10% in olive oil, 1 mL/kg body weight) three times per week, for a total of 10 weeks. We have decided to administrate via i.p. to assure that all animals received the correct dosage, considering that oral administration by gavage for long periods of treatment are stressful to the animals and oral administration by capsules or food may not deliver the same amount of the molecule to all animals. In order to validate the liver fibrosis model through chronic CCl_4 administration used in the present study, we have used Silymarin (SLM) as a positive treatment control, as it is a substance known for its treatment effects in *in vivo* experiments. A SLM dose of 200 mg/kg was administered (Zhang et al., 2018).

Methoxyeugenol treatment was given twice a week, on alternating days with CCl_4 induction, intraperitoneally at 0.25 and 1.0 mg/kg. Doses were selected according to European Food Safety Authority that reports a safety dose for methoxyeugenol ranging from 1 to 30 mg/kg (European Food Safety Authority (EFSA) and Parma, 2011). Therefore, a dose of 1.0 mg/kg was chosen in addition to a lower dose of 0.25 mg/kg, which would not cause any side effect to animals. Animals were euthanized at 24 h after the last administration. Trunk blood was collected by decapitation and the liver was removed and stored. Serum was separated by centrifugation at 5×10^3 rpm. The study was approved by the University (PUCRS) Animal Ethics Committee (CEUA 8318).

For *in vivo* experiments, methoxyeugenol molecule (4-Allyl-2,6-dimethoxyphenol) was administered from the same batch (Sigma-Aldrich - Cat.#W365505/Lot#STBD5682V) used in the *in vitro* experiments, also solubilized in DMSO.

2.2.2. Serum analysis

Blood samples were collected at the end of the experiment and centrifuged at 4 °C. Serum ALT (alanine transaminase), AST (aspartate transaminase), alkaline phosphatase activity and albumin concentration were analyzed by using Labtest Kits (Lagoa Santa, Minas Gerais, Brazil), following the manufacturer's recommendations.

2.2.3. Liver histopathology

After euthanasia, liver samples were fixed with 10% buffered formalin, and embedded in paraffin blocks. Tissues were cut into 4.5 μm sections and stained with hematoxylin and eosin stain (H&E – Cytological Products Soldan, RBP-1700-02A). A semi-quantitative assessment was performed: steatosis (0–3), inflammation (0–2), and ballooning (0–2); according to the Nonalcoholic Fatty Liver Disease Activity Score (NAS) (Kleiner et al., 2005). In order to demonstrate fibrosis, liver sections were stained with Picro Sirius Red. Liver fibrosis also was semi-quantitatively determined: fibrosis (0–4). Images of the

sections were captured through a BMX 43 microscope equipped with a digital DP73 camera (Olympus, Tokyo, Japan). For immunohistochemistry, tissue samples were cut in 4.5 μm thick sections of paraffin-embedded blocks and mounted in slides. Slides were dehydrated and the antigen retrieval was performed in microwave (3 cycles of 5 min each), in citrate buffer pH = 6.0. Samples were washed in PBS (phosphate buffered saline). Peroxidase step (3% in PBS) was performed in slides for 10 min and afterwards washed in PBS. For the blocking step, 5% goat serum in PBS solution with 0.1% of TritonX and incubation at room temperature for 1 h were used. Primary antibodies for αSMA (alpha-smooth muscle actin, #19245, Cell Signaling), NF-kB (nuclear factor kappa B, p-65, #8242, Cell Signaling), p-NF-kB (phosphorylated nuclear factor kappa B, p-p65, #3036, Cell Signaling) and GAPDH (glyceraldehyde 3-phosphate dehydrogenase, #97166, Cell Signaling) in a concentration of 1:200 diluted in blocking solution were incubated at 4 °C overnight. At the following day, samples were washed with PBS and incubated with a secondary anti-rabbit antibody (A16160, Invitrogen), diluted 1:200 in PBS with TritonX 0.1%, for 1 h. After secondary incubation, samples were washed with PBS. DAB (3,3′-diaminobenzidine) was used as chromogen and counterstained with hematoxylin, followed by a rehydration step. Samples were mounted with DPX mountain medium and let to completely dry at room temperature. Images were obtained in a BMX 43 microscope, equipped with DP73 (Olympus, Tokyo, Japan) digital camera. Analysis of stained area were performed by calculation of % area stained with DAB with ImageJ software.

2.3. General techniques

2.3.1. mRNA extraction and real-time qPCR

Gene expression was determined by Real-time qPCR (Applied Biosystems StepOne) in GRX (α-SMA, TGF-β, PPAR-ɣ and PPAR-α) and LX-2 (α-SMA, Col-1(type 1 collagen) and PDGFrβ (platelet-derived growth factor receptor beta) cells treated with methoxyeugenol at 30 μM during 72 h, and also in hepatic tissues from animals submitted to the CCl_4 fibrosis protocol (α-SMA, Col-1, IL-6 (interleukin-6), TNF-α (tumor necrosis factor) and CD 163 (cluster of differentiation 163), iNOS (inducible NO synthase)). mRNA was extracted using TRIzol reagent (Invitrogen, 15596026) and reversely transcribed into cDNA, using the Superscript III SuperMix (Invitrogen, 18080400). All samples had the total mRNA concentration normalized at 5 μg. Relative expression levels of interest genes were performed using the GAPDH as a reference gene. The reactions were catalyzed by using the SYBR Green (Applied Biosystems). Results, expressed as $\Delta\Delta CT^2$, represent the x-fold increase of gene expression compared with the corresponding control group.

2.3.2. Western blot

For protein extraction, liver tissues from mice were homogenized in a solution containing CHAPS 0.5%, β-mercaptoethanol and proteases (Amresco, M221). HSCs LX-2 cells were lysated with a solution containing Triton (Sigma, 9002-93-1), TBS5x, EDTA (ethylenediamine tetraacetic acid) and protease inhibitors. Samples were normalized to 30 μg of protein, separated with electrophoresis (polyacrylamide gel 10% w/v) and transferred to a nitrocellulose membrane (Biorad, 1620112). The blot was washed with Tris-HCl, NaCl, and 0.05% Tween (Sigma, P9416), followed by 30 min incubation in blocking solution TTBS (tween-tris-buffered saline) containing 5% BSA (bovine serum albumin). After, the blot was washed again with TTBS and incubated overnight at 4 °C in blocking solution containing the following primary antibodies: anti-GAPDH, anti-αSMA, anti-NF-kβ (p65) or anti-phosphorylated-NF-kβ (p-p65). After an overnight incubation, the blot was washed and incubated again for 2 h with horseradish peroxidase-conjugated anti-IgG secondary antibody. The band was detected by a gel documentation system (Fujifilm, LAS-3000). Band intensities were quantified through the ImageJ software.

2.3.3. Statistical analysis

Data are reported as mean ± standard deviation (SD) and analyzed by one-way analysis of variance (ANOVA) followed by the Tukey multiple comparison test at a significance level of $p < 0.05$. Otherwise, comparisons were assessed with the non-parametric Mann-Whitney U test. Differences were considered significant at a p-value < 0.05. The statistical program used was the GraphPad Prism Version 5.00.

3. Results

3.1. Methoxyeugenol decreases HSC proliferation without evidence of cytotoxicity

Murine HSC GRX cells treated for 72 h with methoxyeugenol (30, 60, 125 and 250 μM) showed decreased cellular number using the MTT assay (Fig. 1A) and cell counting (Fig. 1B). The investigation of LDH release was performed to determine whether the decrease in the number of GRX cells was due to necrotic cell death. The results showed that all tested concentrations presented released LDH levels similar to the control group (Fig. 1C), indicating that the decrease in the number of cells was not caused by necrosis. In addition, possible effects on cell viability on two other well-known cell lines used for cytotoxicity investigations, VERO - derived from kidney epithelial cells (Fig. 1D), and HepG2 - derived from human hepatocellular carcinoma (Fig. 1E), were investigated. Neither cell lines presented changes in their cellular viability, at the same condition. Thus, based on the minimum concentration for a significant reduction on GRX cell proliferation, 30 μM of methoxyeugenol was selected for the following *in vitro* experiments.

3.2. Methoxyeugenol promotes a quiescent phenotype in HSC

Methoxyeugenol treatment resulted in significant lipid droplets accumulation into the cellular cytoplasm when compared to the vehicle group (Fig. 2A–D). Additionally, methoxyeugenol reduced HSC contractility, as evidenced by significant inhibition of the reduction in collagen gel area (Fig. 2E). Analysis of gene expression in GRX cells treated with methoxyeugenol evidenced significant decrease in profibrotic genes, α-SMA (Fig. 2F) and TGF-β (Fig. 2G), an overexpression of PPAR-ɣ (Fig. 2H), and no changes in PPAR-α (Fig. 2I) compared to vehicle-treated cells. Importantly, deactivation properties of methoxyeugenol in murine HSC cell line were validated in primary HSC isolated from CCl_4-induced cirrhotic rats (Fig. S1).

3.3. Methoxyeugenol protects HepG2 hepatocytes from oxidative stress damage

Methoxyeugenol exhibited strong basal antioxidant capacity, as demonstrated by the DPPH assay (Fig. 3A). Its antioxidant capacity was maintained under high oxidative stress conditions, as those produced by CCl_4 *in vitro* (Fig. 3B). In addition, cell damage was also verified in HepG2 cells that received CCl_4 by measuring LDH release in the supernatant, demonstrating that methoxyeugenol prevented LDH release (Fig. 3C).

3.4. Methoxyeugenol decreases liver injury, inflammation and fibrosis in vivo

Animals treated with CCl_4 showed an increase in lobular inflammation score compared to the vehicle group in an effect prevented in the animals receiving methoxyeugenol (Fig. 4). The effects of methoxyeugenol preventing liver injury was further confirmed by significant reduction in ALT serum levels (Fig. 4E), with no changes in albumin, alkaline phosphatase and AST levels (Fig. S2). Expression of pro-inflammatory genes (IL-6, TNF-α, iNOS and IL-8) was significantly increased in the CCl_4 group compared to both control and methoxyeugenol treated groups (Fig. 4G-M). The NF-kB protein expression

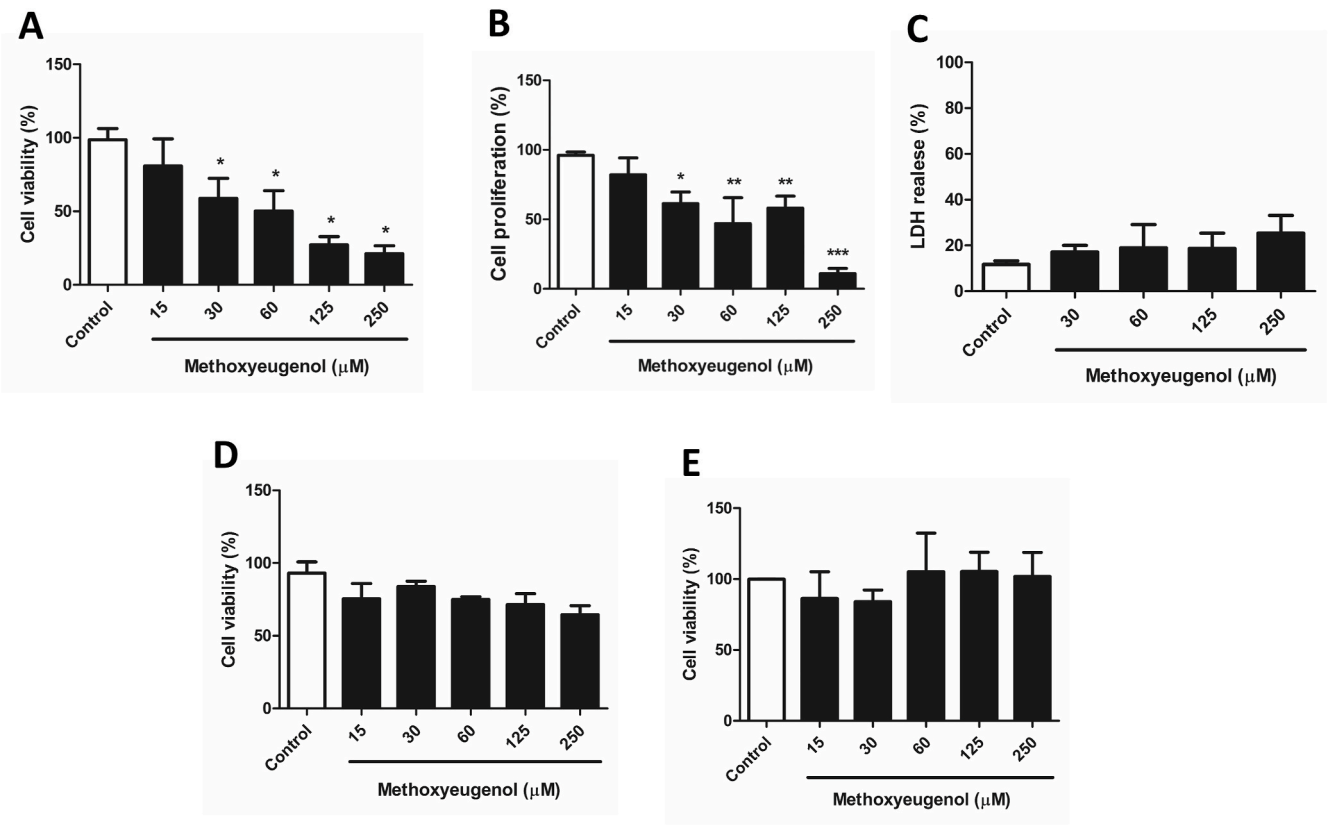

Fig. 1. Effects of methoxyeugenol on cell viability. Effects of methoxyeugenol were determined at concentrations of 15, 30, 60, 125 and 250 μM, during 72 h of treatment, in GRX cells through MTT (A); cell counting (B); and LDH release in the supernatant (C). Cellular viability was also evaluated by MTT assay in endothelial cells from VERO strain (D) and hepatocytes HepG2 cells (E) at concentrations of 30, 60, 120 and 250 μM. Data represent mean ± standard deviation (SD) (n = 4). *$p < 0.05$ compared with control. **$p < 0.01$ compared with control. ***$p < 0.001$ compared with control.

showed a similar result, as animals treated with CCl_4 presented a greater expression ratio than animals from both control and methoxyugenol treated groups (Fig. 4I). The protein CD163, a cluster of differentiation of M2 macrophages, was significantly induced in animals treated with CCl_4 and this effect was attenuated by methoxyeugenol (Fig. 4J).

Liver sections were stained with H&E and Picro Sirius Red and scores for ballooning, steatosis and fibrotic areas were analyzed. The CCl_4 treated group exhibited increased steatosis, ballooning, Sirius red-stain and α-SMA area in comparison to the vehicle group (Fig. 5A–F), confirming that animals developed a fibrotic process. Treatment with methoxyeugenol was able to attenuate all the analyzed parameters, both at 0.25 and 1.0 mg/kg (Fig. 5D–G). Levels of mRNA expression of α-SMA and Col-1 genes were evaluated in the hepatic tissue and animals treated with CCl_4 showed a high expression ratio of Col-1 and α-SMA genes, while methoxyeugenol treated animals maintained the expression rate similar to the control group (Fig. 5H–I). In addition, treatment with SLM also decreased the liver fibrosis development in the same experimental model (Fig. S3).

3.5. *Methoxyeugenol improves human HSCs phenotype via PPAR-ɣ activation*

Similarly to murine HSC, methoxyeugenol reduced the expression of α-SMA in human HSC LX-2 cells (Fig. 6A). In order to assess the role of PPAR-ɣ in this beneficial effect, cells were pre-treated with a specific PPAR-ɣ antagonist (GW9662) for 24 h, and then treated with methoxyeugenol or vehicle for 72 h. Results demonstrated that the effect of methoxyeugenol in LX-2 cells was suppressed when PPAR-ɣ was antagonized, exhibiting no improvement in the expression of Col1a1 (Fig. 6B) and α-SMA (Fig. 6C) in comparison to the control group.

4. Discussion

Chronic damage in liver tissue triggers a regenerative process, which leads to hepatic fibrosis development, characterized as a continuous healing response with hepatocytes and HSCs playing a central role. Hepatocytes injury may trigger HSCs to an activated phenotype by an inflammatory signaling pathway, guided either indirectly by Kupffer cells or directly by the release of growth factors and pro-inflammatory cytokines. A previous study reported the beneficial effects of nutraceutical supplementation in liver diseases, such as steatosis, metabolic syndrome and liver fibrosis (Bravo, 2009). Molecules as caffeine, resveratrol, curcumin, vitamin E and quercetin have shown positive effects against oxidative stress, inflammation and HSCs activation (Li et al., 2017). Nevertheless, liver fibrosis and its clinical complications still represent an important clinical problem and therefore novel therapeutic approaches are needed. Our objective was to evaluate the effects of methoxyeugenol in the regulation of activated HSCs phenotype and in the protection of hepatocytes from oxidative stress, as well as its protective effects on hepatic fibrosis.

Our *in vitro* results demonstrated that methoxyeugenol promotes HSCs deactivation, defined as reduced proliferation and lower expression of phenotypic activation markers. Indeed, treatment with methoxyeugenol inhibited the increment in cultured GRX cell number, without evidence of cell death, which suggests a reduction in the proliferative rate. Possible toxicity of the compound was discarded by analyzing the expression of cell death markers and these effects were further corroborated by results from two other cell lines used as internal controls. Vero cells of epithelial origin are commonly used in assays to evaluate cytotoxicity, as well as the HepG2 cell line, which has a carcinogenic origin, but maintain many similarities to human hepatocytes.

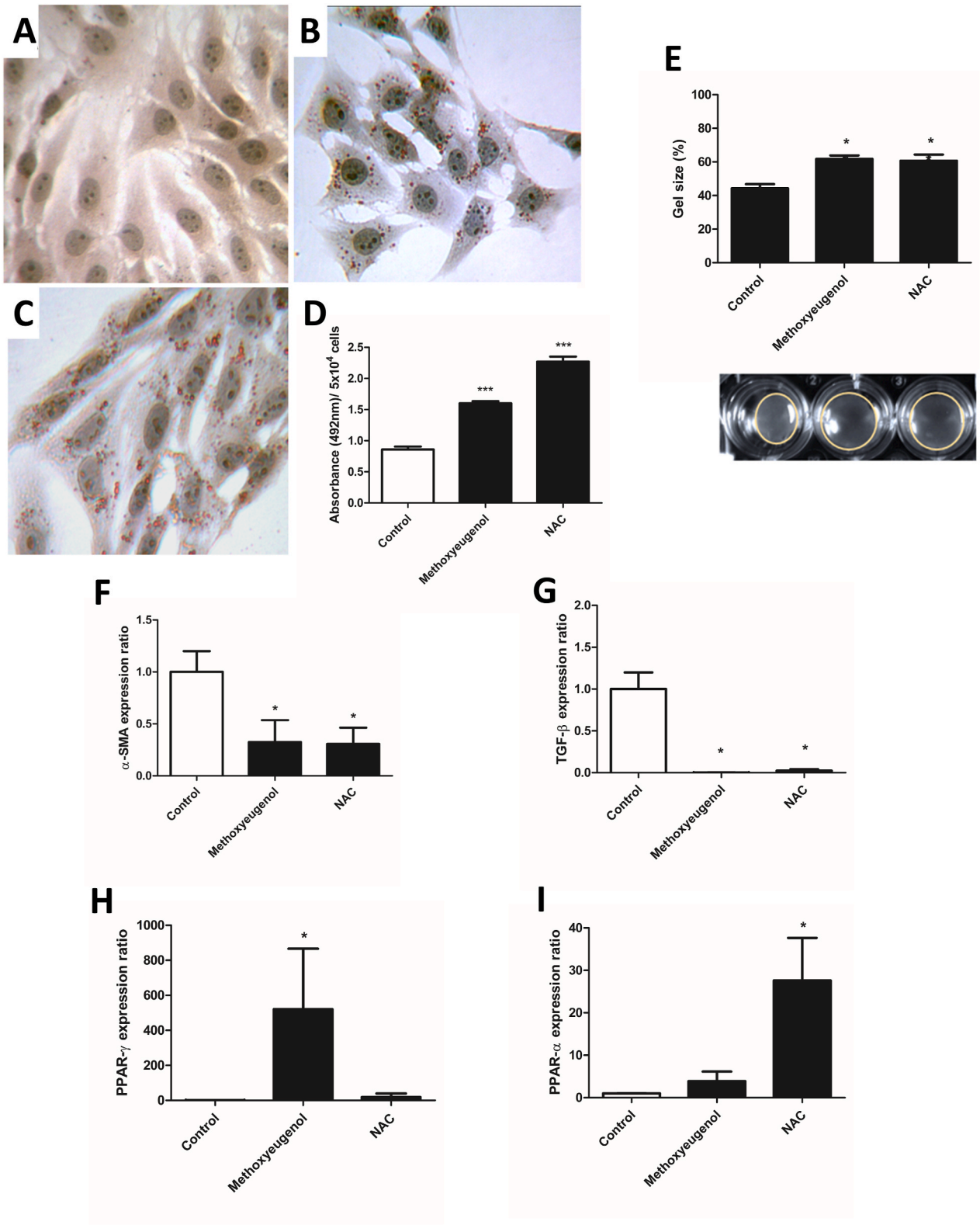

Fig. 2. Evaluation of HSCs activation markers. Lipid droplets staining and cellular contraction. Oil Red-O lipid staining of GRX cells (A–D). Control (A), methoxyeugenol at 30 μM (B), NAC at 2.5 mM (C) after 72 h; 400× magnification. Lipid quantification (D) are shown as the absorbance (492 nm) value obtained for Oil Red adjusted for the number of cells (5×10^4). Cellular contraction of GRX cells was evaluated by measuring the gel area after treatment with methoxyeugenol. Values represent the percentage occupied by the gel relative to the total area of the well (E). Data represent mean ± standard deviation (SD) (n = 4). *$p < 0.05$ compared with control. ***$p < 0.001$ compared with control. Expression of mRNA ratio of α-SMA (F), TGF-β (G), PPAR-ɣ (H) and PPAR-α (I) in GRX cells treated with methoxyeugenol at 30 μM during 72 h. NAC was used as a positive control at 2.5 mM. Data represent mean ± standard deviation (SD) (n = 4). *$p < 0.05$ compared with control. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

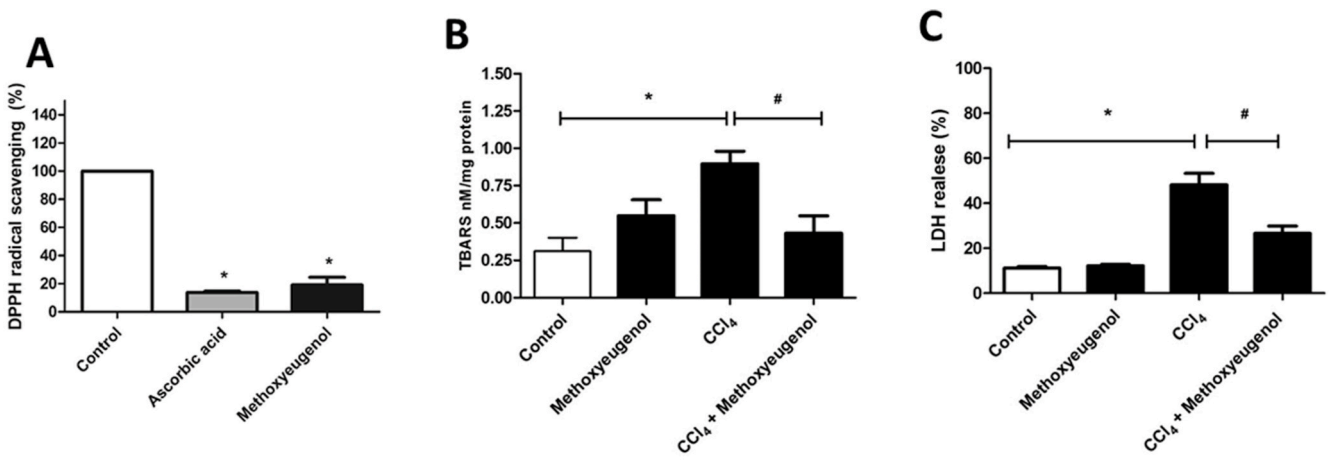

Fig. 3. Antioxidant effects of methoxyeugenol. Methoxyeugenol (30 μM) antioxidant activity was evaluated by DDPH scavenging assay and the ascorbic acid (550 μg/mL) was used as a positive control (A). Oxidative stress damage on hepatocytes challenged with CCl_4 for 6 h were determined by the measurement of TBARS (B) and by the quantification of percentage of LDH release in cell culture supernatant (C). Data represent mean ± standard deviation (SD) (n = 4). $*p < 0.05$ compared with control. $\#p < 0.05$ compared with the CCl_4 group.

In order to determine the possible effects of the compound in the HSCs phenotype, expression levels of profibrotic genes TGF-β and α-SMA were investigated. Results have shown that the mRNA expression of these genes was suppressed by treatment with methoxyeugenol. The cytokine TGF-β is a growth factor that plays an important role in the development of liver fibrosis, particularly by activating quiescent HSCs, which loses the characteristic of accumulating vitamin A in its cytoplasm and additionally enhances its contractibility (Hernandez-Gea and Friedman, 2011). The contraction capacity of HSC is related to increased expression of α-SMA, since this actin isoform is highly expressed in myofibroblasts and their activation play a key role in the development of the fibrotic response. Collagen gel test confirmed the decrease of contraction capacity of HSC cells treated with methoxyeugenol. In addition, in human activated HSCs (LX-2), the treatment reduced mRNA expression levels of α-SMA and Collagen1, as well as protein levels of α-SMA, indicating a modulating effect of methoxyeugenol on activated HSCs phenotype.

HSCs are the main storage source of vitamin A in the human body, which is located in quiescent HSCs cytoplasm, into lipid droplets. The loss of cytoplasmatic lipid accumulation is a morphological characteristic of activated HSCs. The maintenance of the lipid metabolism occurs by nuclear receptors of the PPAR's family, mainly by PPAR-α/ɣ (Chen et al., 2015). The treatment with methoxyeugenol increased PPAR-ɣ mRNA expression, which associated with the results obtained with Oil Red-O staining, indicates a more quiescent phenotype by lipid droplets formation in GRX cells. In addition, treatment with NAC also induced lipid droplets development in the cytoplasm, although this seems to be through a PPAR-α activation pathway (Kim et al., 2001). More importantly, the pre-treatment of LX-2 cells with specific PPAR-ɣ antagonist (GW9662) suppressed the effects of methoxyeugenol treatment on activated HSCs phenotype markers collagen1 and α-SMA. Taken together, these results suggest that the beneficial effects of methoxyeugenol in murine and human HSCs is, at least in part, through the activation of PPAR-ɣ. Our results are in agreement with previous studies using synthetic activators of PPARs, although the present study demonstrates such effects using a natural compound that may present much lower undesired side-effects then full agonists of PPARs (Chen et al., 2015; Nan et al., 2013). Several studies have shown that the PPAR-γ activation can inhibit macrophage activation and inflammatory pathways mediated by NF-kβ (Mirza et al., 2019; Qu et al., 2017). The PPAR-γ activation may improve the phenotype modulation of activated HSC by restoring the lipid metabolism and by inhibiting the inflammatory signaling. These combined effects show that natural activators of PPARs are great alternatives for the development of new therapies for chronic liver diseases.

Moreover, we have investigated the potential of methoxyeugenol to prevent hepatocyte injury *in vitro*. One important pathway for HSCs activation is through hepatocyte signaling, since these cells represent approximately 80% of the hepatic cell population and are responsible for metabolizing many substances. The damage to the liver tissue is often due to oxidative stress derived from metabolism or inflammatory processes. Therefore, the ability of methoxyeugenol to scavenge free radicals was evaluated. Results revealed that methoxyeugenol has high antioxidant activity, both under basal *in vitro* conditions and upon oxidative stress challenge achieved through CCl_4 treatment.

In summary, the *in vitro* results demonstrated that methoxyeugenol were able to deactivate human and murine HSC, and to protect hepatocytes. Therefore, *in vivo* activity of methoxyeugenol in CCl_4-induced liver fibrosis model was investigated. The periodic administration of CCl_4 leads to lipid accumulation (steatosis), increased inflammatory infiltrate, loss of normal hepatocytes, collagen deposition, and fiber segmentation formation. These hepatic tissue alterations can be classified by the degree of hepatic fibrosis according to NAFLD Activity Score in HE stained sections. Interestingly, treatment with methoxyeugenol attenuated the inflammatory process and fibrosis observed in mice chronically treated with CCl_4. In addition, treatment with methoxyeugenol was able to decrease ALT serum levels when compared to the vehicle group. Exacerbated deposition of extracellular matrix components during liver fibrosis is a result of HSC activation and related to the expression of type 1 collagen and α-SMA genes. Both genes and α-SMA protein expression were suppressed with methoxyeugenol treatment. Moreover, in the same experimental model, we showed that the use of SLM as a positive control decreases the liver fibrosis development.

Inflammation is a beneficial process for the regeneration of damaged tissues and the elimination of pathogens, however chronic inflammation may result in a permanent healing state in the liver, initiating the fibrotic process. NF-kB is a nuclear transcription factor involved in inflammatory regulation and cell death in several cell types. NF-kB is a heterodimer consisting of two subunits, p65 and p50. When not stimulated, the NF-kB factor is found in the cytoplasm, linked to the Ik-B inhibitory protein. This complex prevents the translocation of the NF-kB to the nucleus. The phosphorylation of Ik-B, a family of inhibitory proteins, by Ik-B kinases in critical serine residues (Ser32 and Ser36) determines the release of NF-kB to the cell nucleus, where it will play its role as a factor of transcription for inflammatory interleukins. Thus, phosphorylation and degradation of Ik-B are necessary for translocation to occur. Different stimuli can activate NF-kB, including pathogen-related molecules, such as LPS (lipopolysaccharide), or inflammatory

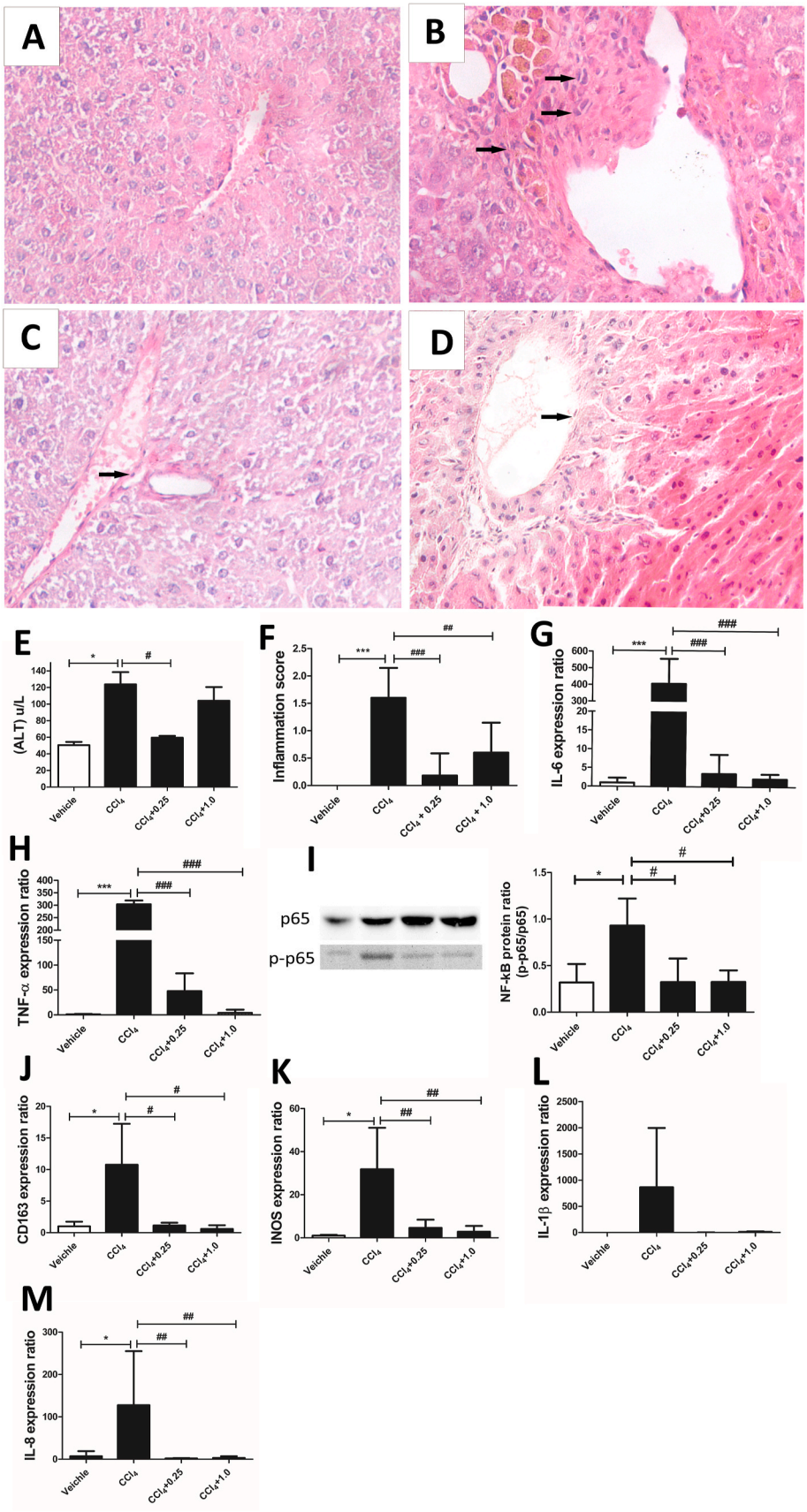

Fig. 4. *In vivo* effects of methoxyeugenol on liver inflammation. Lobular inflammation was evaluated on H&E staining of mice treated with vehicle (A), CCl_4 (B), methoxyeugenol 0.25 mg/kg + CCl_4 (C) or methoxyeugenol 1.0 mg/kg + CCl_4 (D). Black arrows indicate hepatic infiltrates. Magnification of 400×. ALT serum levels (E), quantification of lobular inflammation score (F), IL-6 (G), TNF-α (H) and protein expression ratio of NF kβ (p-p65/p65) (I). Expression of mRNA ratio of CD163 (J), INOS (K) IL-1b (L), IL-8 (M). Data represent mean ± standard deviation (SD) (n = 3–8). $*p < 0.05$ compared with the vehicle group. $**p < 0.01$ compared with the vehicle group. $***p < 0.001$ compared with the vehicle group. $\#p < 0.05$ compared with the CCl_4 group. $\#\#p < 0.01$ compared with the CCl_4 group. $\#\#\#p < 0.001$ compared with the CCl_4 group.

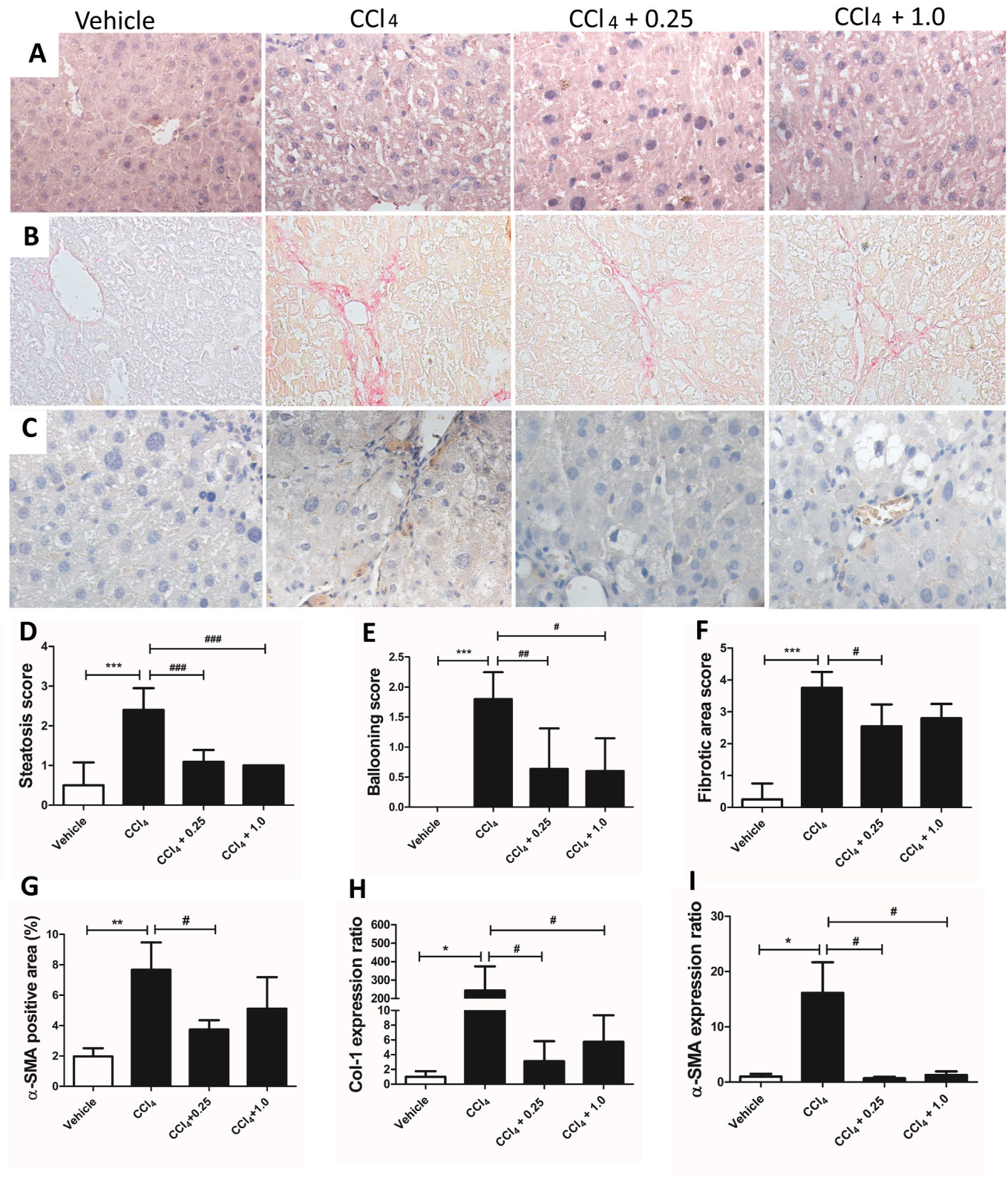

Fig. 5. ***In vivo* effects of methoxyeugenol on liver fibrosis**. H&E (A), Picro Sirius (B), α-SMA immunohistochemistry (C) staining of representative mice treated with vehicle, vehicle + CCl_4, methoxyeugenol 0.25 mg/kg + CCl_4 or methoxyeugenol 1.0 mg/kg + CCl_4. Magnification of 400×. Quantification of Steatosis score (D), Ballooning score (E), Fibrotic area score (F), % of positive α-SMA area (G), expression of mRNA ratio of Col-1 (H) and α-SMA (I). Data represent mean ± standard deviation (SD) (n = 8). *$p < 0.05$ compared with the vehicle group. ***$p < 0.001$ compared with the vehicle group. #$p < 0.05$ compared with the CCl_4 group. ##$p <$ 0.01 compared with the CCl_4 group. ###$p < 0.001$ compared with the CCl_4 group.

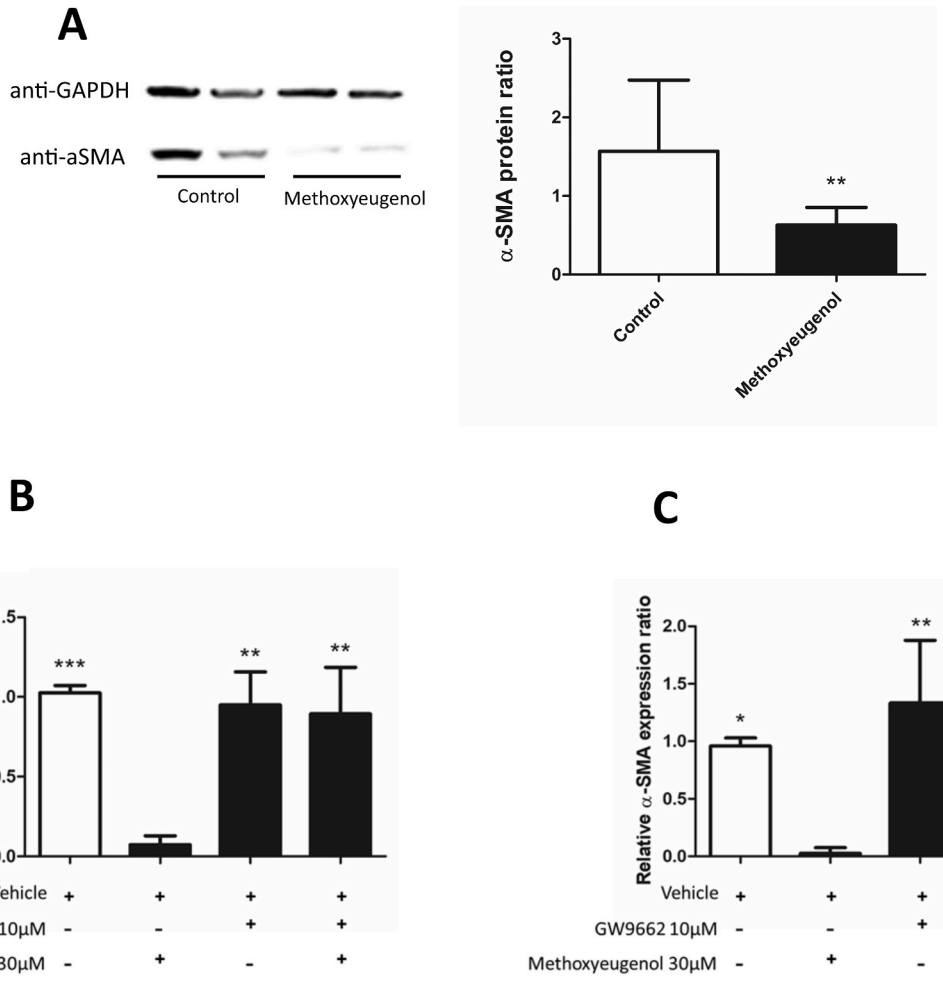

Fig. 6. Effect of PPAR-ɣ antagonist pre-treatment on human activated HSC. Protein expression ratio of α-SMA (A) in LX-2 cells treated with methoxyeugenol for 72 h. Evaluation of activation markers of HSC on LX-2 cells pre-treatment with GW9662 10 μM for 24 h, followed by treatment with methoxyeugenol 30 μM or vehicle for 72 h, Col1a1 (B) and α-SMA (C). Data represent mean standard deviation (SD) (n = 3). *$p < 0.05$ compared with the methoxyeugenol group. **$p < 0.01$ compared with the methoxyeugenol group. ***$p < 0.001$ compared with the methoxyeugenol group.

cytokines, such as tumor necrosis factor-α (TNF-α) (Luedde and Schwabe, 2011). NF-kB activation (phosphorylation) leads to transcription of many genes related to inflammatory cytokines, such as iNOS and interleukins (IL-1b, IL-6, IL-8). NF-kB activation also leads to the recruitment of inflammatory infiltrates that are characterized by intralobular inflammation, which consists of a variety of inflammatory cells (lymphocytes, neutrophils, eosinophils, and Kupffer cells). Even though the inflammatory role of NF-kB is not completely elucidated, it is known that its basal levels are beneficial for maintaining liver tissue homeostasis.

Moreover, studies have shown that NF-kB deletion may lead to spontaneous development of liver fibrosis (Luedde and Schwabe, 2011). However, pronounced activation of NF-kB may also result in the development of fibrosis, considering that its activation leads to HSC activation. Even with this dual role of NF-kB, drugs that influence the regulation of inflammation by one of its pathways are considered as research candidates for the development of new therapies (Papa et al., 2009). Treatment with methoxyeugenol was able to reduce intralobular inflammation, decrease TNF-α, iNOS, IL-6, and IL-8 gene expression, as well as NF-kB protein expression. Methoxyeugenol increases the expression of PPAR-ɣ, which is considered an important factor in the metabolism of lipids and glucose, also participating in several biological responses, such as anti-inflammatory and antiproliferative actions. Its anti-inflammatory effects cause inhibition of NF-kB and consequent suppression of the expression of pro-inflammatory proteins, including IL-6, IL-8, TNF-α, and MCP-1 (Jung-Hoon et al., 2015). The hemoglobin scavenger receptor, CD163, is a M2 macrophage-specific protein and higher CD163 expression in macrophages is characteristic of tissues responding to inflammation (Etzerodt and Moestrup, 2012). The treatment with methoxyugenol decreased the inflammation *in vivo* and the CD163 results have also shown a decrease, indicating that there is less scavenger process.

In order to validate the effects on human HSC and to investigate the PPAR-ɣ role in the use of methoxyeugenol, we have used the irreversible and selective PPAR-ɣ inhibitor, GW9662. The use of GW9662 on cell lines, aiming to test PPAR-ɣ potential ligands are well established on previous studies, normally using the concentration of 10 μm (Ma et al., 2017). The pre-treatment of LX-2 cells with the specific PPAR-ɣ antagonist (GW9662) suppressed the effects of methoxyeugenol treatment on activated HSC phenotype markers collagen1 and α-SMA. Regarding HSC activation, the PPAR-ɣ expression is dramatically reduced, whereas the opposite effect is observed on HSC quiescent phenotype, demonstrating that PPAR-ɣ is an important modulator of HSC activation (He et al., 2019). In addition, studies have shown that an increase in PPAR-ɣ expression leads to a NF-κB suppression (Zong et al., 2013). Thus, the regulation of this nuclear receptor can bring different benefits for liver fibrosis treatment, modulating HSC phenotype and reducing inflammatory signaling.

5. Conclusions

In conclusion, the present study has shown the use of a nutraceutical molecule, which is currently mainly used as food additive, as a new therapeutic agent for the treatment of hepatic fibrosis. Results showed that methoxyeugenol was able to modulate the activated phenotype of HSC by activating PPAR-ɣ and demonstrated a protective effect against oxidative stress damage in hepatocytes. *In vivo* experiments further indicated that methoxyeugenol promotes a protective effect, improving inflammatory parameters and fibrosis, probably due to an increase of PPAR-ɣ activation that inhibits the NF-kB inflammatory signaling.

Declaration of competing interest

All the authors have no conflict of interest and all of them have read the manuscript and approved the submitted manuscript.

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Ethics approval

All procedures performed in this study involving animals were approved by PUCRS Animal Ethics Committee, code 8318.

Author contributions

Conceptualization, Bruno de Souza Basso; Formal analysis, Bruno de Souza Basso; Funding acquisition, Jordi Gracia-Sancho and Jarbas Rodrigues de Oliveira; Investigation, Bruno de Souza Basso, Gabriela Viegas Haute, Martí Ortega Ribera, Carolina Luft, Géssica Luana Antunes, Matheus Scherer Bastos, Eduardo Cassel, Eliane Romanato Santarém, Jordi Gracia-Sancho and Jarbas Rodrigues de Oliveira; Methodology, Bruno de Souza Basso, Gabriela Viegas Haute, Martí Ortega Ribera, Carolina Luft, Géssica Luana Antunes, Matheus Scherer Bastos, Leonardo Pfeiff Carlessi, Vitor Giancarlo Levorse, Eliane Romanato Santarém, Jordi Gracia-Sancho and Jarbas Rodrigues de Oliveira; Resources, Eliane Romanato Santarém; Writing – original draft, Bruno de Souza Basso; Writing – review & editing, Bruno de Souza Basso, Márcio Vinícius Fagundes Donadio, Eliane Romanato San-tarém, Jordi Gracia-Sancho and Jarbas Rodrigues de Oliveira.

CRediT authorship contribution statement

Bruno de Souza Basso: Conceptualization, Formal analysis, Investigation, Methodology, Writing – original draft, Writing – review & editing. **Gabriela Viegas Haute:** Investigation, Methodology. **Martí Ortega-Ribera:** Investigation, Methodology. **Carolina Luft:** Investigation, Methodology. **Géssica Luana Antunes:** Investigation, Methodology. **Matheus Scherer Bastos:** Investigation, Methodology. **Leonardo Pfeiff Carlessi:** Methodology. **Vitor Giancarlo Levorse:** Methodology. **Eduardo Cassel:** Investigation. **Márcio Vinícius Fagundes Donadio:** Writing – review & editing. **Eliane Romanato Santarém:** Investigation, Methodology, Resources, Writing – review & editing. **Jordi Gracia-Sancho:** Funding acquisition, Investigation, Methodology, Writing – review & editing. **Jarbas Rodrigues de Oliveira:** Funding acquisition, Investigation, Methodology, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jep.2021.114433.

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