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Extracts of *Lindera obtusiloba* induce antifibrotic effects in hepatic stellate cells via suppression of a TGF-β-mediated profibrotic gene expression pattern☆

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Abstract

Liver fibrosis is characterized by high expression of the key profibrogenic cytokine transforming growth factor (TGF)-β and the natural tissue inhibitor of metalloproteinases (TIMP)-1, leading to substantial accumulation of extracellular matrix. Liver fibrosis originates from various chronic liver diseases, such as chronic viral hepatitis that, to date, cannot be treated sufficiently. Thus, novel therapeutics, for example, those derived from Oriental medicine, have gained growing attention. In Korea, extracts prepared from *Lindera obtusiloba* are used for centuries for treatment of inflammation, improvement of blood circulation and prevention of liver damage, but experimental evidence of their efficacy is lacking. We studied direct antifibrotic effects in activated hepatic stellate cells (HSCs), the main target cell in the fibrotic liver. *L. obtusiloba* extract (135 μg/ml) reduced the de novo DNA synthesis of activated rat and human HSCs by about 90%, which was not accompanied by cytotoxicity of HSC, primary hepatocytes and HepG2 cells, pointing to induction of cellular quiescence. As determined by quantitative polymerase chain reaction, simultaneous treatment of HSCs with TGF-β and *L. obtusiloba* extract resulted in reduction of TIMP-1 expression to baseline level, disruption of the autocrine loop of TGF-β autoinduction and increased expression of fibrolytic matrix metalloproteinase (MMP)-3. In addition, *L. obtusiloba* reduced gelatinolytic activity of HSC by interfering with profibrogenic MMP-2 activity. Since *L. obtusiloba* extract prevented intracellular oxidative stress experimentally induced by *tert*-butylhydroperoxide, we concluded that the direct antifibrotic effect of *L. obtusiloba* extract might be mediated by antioxidant activity.

Thus, *L. obtusiloba*, traditionally used in Oriental medicine, may complement treatment of chronic liver disease.

Keywords: Plant extracts; *L. obtusiloba*; Hepatic stellate cells; Liver fibrosis; Oxidative stress

Abbreviations: α-SMA, alpha smooth muscle actin; CI, collagen I; CM-H2DCFDA, 5-(6-)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate acetylester; FBS, fetal bovine serum; GAPDH, glyceraldehyde phosphate dehydrogenase; HSC, hepatic stellate cell; IC_{50}, half-maximum inhibitory concentration; LDH, lactate dehydrogenase; MMP, matrix metalloproteinase; ROS, reactive oxygen species; TGF, transforming growth factor; TIMP, tissue inhibitor of metalloproteinases; APMA, 4-aminophenyl mercuric acetate; PCR, polymerase chain reaction.

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1. Introduction

Liver cirrhosis is among the leading causes of morbidity and mortality in industrialized countries, and approved nonsurgical therapies to effectively prevent liver fibrosis or to treat established cirrhosis are not available. Liver fibrosis is the uniform response of various chronic liver diseases including chronic infection with hepatitis B and C virus, alcohol consumption, insulin resistance (nonalcoholic fatty liver disease), iron storage, autoimmune dysfunction and others [1]. Herein, a chronic scarring process characterized by an excess of extracellular matrix synthesis and its decreased degradation results in the accumulation of matrix molecules, particularly collagens. It is generally accepted that the sinusoidal hepatic stellate cell (HSC) is the most relevant cell type in the pathogenesis of liver fibrosis [2]. HSCs become activated by various stimuli, for example, by cytokines including platelet-derived growth factor and transforming growth factor (TGF)-β that induce proliferation and an up to 10-fold increased synthesis of fibril-forming collagen I (CI) [3]. Activated HSCs produce both TGF-β and tissue inhibitors of metalloproteinases (TIMPs), which lead to enhanced extracellular matrix synthesis and decreased matrix degradation, respectively [2]. Therefore, HSCs constitute a prime target for antifibrotic therapy [4,5]. Antifibrotics are classified by their mode of action as suppressors of hepatic inflammation, as promoters of matrix degradation or as antioxidants reducing the profibrotic impact of reactive oxygen species (ROS) on HSC [1].

Oriental medicine traditionally uses compositions of different plants and plant ingredients to prevent or cure liver diseases [6]. Evidence from several studies suggest that certain herbal combinations derived from traditional Chinese medicine and Japanese Kampo medicine such as Inchin-ko-to, Sho-saiko-to and Compound 861 carry antioxidant and possibly antifibrotic potential, which could be exploited for the treatment of chronic fibrosing liver diseases [7–9]. In this context, a combination of Western and Oriental medicine-based therapies may exert a synergistic beneficial activity and even diminish adverse side effects associated with interferon-α-based treatments [10,11]. However, only few randomized prospective studies were performed, for example, with Sho-saiko-to, suggesting a possible preventive effect in terms of development of liver fibrosis and hepatoma [12,13]. Chemically, bioactive components isolated from the Japanese spice bush *Lindera obtusiloba* belong to the groups of lignans and butanolides and were shown to exert antitumor activity by exhibiting cytotoxicity against cultured human tumor cell lines A549 (non-small-cell lung cancer), SK-OV-3 (ovarian cancer), SK-MEL-2 (skin cancer), XF498 (CNS cancer) and HCT15 (colon cancer) with half-maximum inhibitory concentration (IC_{50}) values ranging from 3 to 20 μg/ml [14,15]. In traditional Chinese and Korean medicine, *L. obtusiloba* is used for treatment of inflammation and improvement of blood circulation [16], and in Korea, herbal infusions of *L. obtusiloba* are applied to treat chronic liver diseases (K. Kim, personal communication). However, so far, no clinical studies were published. Furthermore, no data are available as to whether *L. obtusiloba* may harbor antifibrotic activity.

This study investigated direct effects of decoctions of wood and bark from *L. obtusiloba* on cell proliferation, cytotoxicity, the expression of fibrosis-related genes, matrix metalloproteinase (MMP) activity and oxidative stress in HSC and liver protective effects of the extract on freshly isolated hepatocytes and HepG2 cells.

2. Materials and methods

2.1. Cell lines, cell culture and isolation of primary hepatocytes

CFSCs isolated from rats treated with carbon tetrachloride [17] were obtained from W. Dieterich (Erlangen, Germany). Human HSCs freshly outgrown from liver tissue biopsies were kindly provided by M. Pinzani (Florence, Italy) [18]. The hepatoma cell line HepG2 (ATCC HB-8065), a standard cell line for liver cytotoxicity studies [19], was purchased from LGC Promochem (Wesel, Germany). All cell lines were routinely maintained in standard culture medium consisting of Dulbecco's modified Eagle's medium for basal medium supplemented with 10% fetal bovine serum (FBS) and 1 μl/ml gentamicin (Invitrogen, Groningen, Netherlands). Primary hepatocytes were freshly isolated from a male Sprague–Dawley rat (450 g) after anesthesia with pentobarbital (50 mg/kg of body weight ip) by a two-step portal collagenase perfusion of the liver and Percoll density-gradient centrifugation as previously described [20]. Cell viability was assessed by trypan blue dye exclusion. Hepatocytes were resuspended in Williams' E medium supplemented with 10% heat-inactivated FBS and subsequently seeded on plastic dishes coated with rat-tail collagen. After allowing cells to adhere for 2 h, unattached cells were poured off and medium was replaced by serum-free medium. Cells were allowed to rest for 24 h before exposure to different concentrations of *L. obtusiloba* (0–400 μg/ml) for additional 24 h.

2.2. L. obtusiloba extracts

Dried wood and bark from twigs of *L. obtusiloba* (20 g) were simmered in 1.8 L distilled water for 4 h. Solids were discarded after filtration, and the clear supernatant (1.5 L) was lyophilized (at Wonkwang University, Iksan, Korea; 1 to 1.5 L of the tea corresponds to the daily amount applied to liver patients). For long-term storage, the resulting 500 mg powder was kept at 4°C. To obtain stock solutions of *L. obtusiloba* extract, the powder (10 mg/ml) was allowed to dissolve in distilled water at 60°C for 30 min. Final concentrations corresponded

Table 1
Probes and primers for quantitative real-time PCR targeting rat cDNA

Target	Oligonucleotide sequence (5′–3′)		
	Probe[a]	Primer sense	Primer antisense
CI	TTCTTGGCCATGCGTCAGGAGGG	TCCGGCTCCTGCTCCTCTTA	GTATGCAGCTGACTTCAGGGATGT
TGF-β1	ACCGCAACAACGCAATCTATGACAAAACCA	AGAAGTCACCCGCGTGCTAA	TCCCGAATGTCTGACGTATTGA
TIMP-1	TTCTGCAACTCGGACCTGGTTATAAGG	TCCTCTTGTTGCTATCATTGATAGCTT	CGCTGGTATAAGGTGGTCTCGAT
α-SMA	CTCGGCCGCCTGCTTCACCA	CCTGCCAAGTATGATGACATCAAGA	GTAGCCCAGGATGCCCTTTAGT
MMP-3	AGATGGTATTCAATCCCTCTATGGACCTCC	CCGTTTCCATCTCTCTCAAGATGA	CAGAGAGTTAGATTTGGTGGGTACCA
MMP-13	TCTGGTTAGCATCATCATAACTCCACACGT	GGAAGACCCTCTTCTTCTCA	TCATAGACAGCATCTACTTTGTC
GAPDH	TGGTGAAGCAGGCGGCCGAG	CCTGCCAAGTATGATGACATCAAGA	GTAGCCCAGGATGCCCTTTAGT

[a] All probes were labeled 5′ with 6-carboxy-fluorescein and 3′ with 6-carboxy-tetramethylrhodamin.

to 400 mg/ml of the original twig amount. All concentrations given in the text refer to the dissolved powder. Aliquots were stored at −20°C.

2.3. Cell proliferation, morphology and viability/cytotoxicity

Microcultures of 5×10^3 rat CFSC and human HSC, respectively, were set up in 96-well tissue culture plates (Nunc, Roskilde, Denmark) in 100 μl medium with 5% FBS. After 24 h, medium was discarded and cell layers were washed with basal medium before 100 μl culture medium containing 0.1% FBS was added to each well. After additional 24 h, cultures were treated with dilutions of *L. obtusiloba* extracts and FBS as indicated. Proliferation was determined using [^{3}H]thymidine (GE Healthcare, Munich, Germany) uptake for 4 h after an 18-h incubation period. DNA from cells fixed with 10% trichloroacetic acid was solubilized with 200 mmol/L sodium hydroxide. The solution was neutralized by an equal volume of 800 mmol/L hydrochloric acid and transferred to glass filter pads. Radioactive decay was monitored by liquid β-scintillation counting (LKB Wallac, Turku, Finland). Cell morphology was assessed by light microscopy (Olympus, Hamburg, Germany) after sulforhodamine B staining as previously described [21]. Cell viability/cytotoxicity was determined by conversion of 4-methylumbelliferyl heptanoate (Sigma, Deisenhofen, Germany) to fluorescent 4-methylumbelliferone (λ_{ex}: 355 nm, λ_{em}: 460 nm) [22]. Fluorescence was determined using a SpectraMax Gemini EM 96-well microplate fluorometer (Molecular Devices, Sunnyvale, CA) and 96-well black microplates (Greiner Bio-One, Frickenhausen, Germany). Cytotoxicity of *L. obtusiloba* was assessed by trypan blue dye exclusion in freshly isolated rat hepatocytes. After subsequent trypsinization, cells were stained with 0.4% trypan blue (Sigma) for 5 min. Trypan-blue-excluding cells were counted and morphology was visualized under a light microscope. Lactate dehydrogenase (LDH) leakage was assayed in the supernatants of treated and nontreated cells with an automated biochemical analyzer (COBAS MIRA plus, Hoffmann-La Roche, Basel, Switzerland) using an enzymatic method. The release of LDH into the medium from cells represents a change in cell membrane permeability.

2.4. Assessment of apoptosis by caspase-3/7 activity

Cultures of 2×10^5 HepG2 cells or CFSC were set up in six-well tissue culture plates (Nunc) with standard culture medium. Confluent cell monolayers were thoroughly washed with basal medium and subsequently incubated with culture medium containing 0.1% FBS for 24 h. *L. obtusiloba* extracts (135 μg/ml) and/or staurosporine (100 nmol/L) was supplemented for an additional 24 h with 5% or 0.1% FBS in the culture medium for HepG2 cells and CFSC, respectively. Apoptosis was determined by caspase-3/7 expression according to the manufacturer's instructions (SensoLyte Homogenous AFC Caspase-3/7 Assay Kit, AnaSpec, San Jose, CA). In brief, cells were lysed in 300 μl lysis buffer for 1 h at 4°C. The clear supernatant obtained after centrifugation at 2500×*g* for 30 min was stored at −80°C until measurement. Caspase-3/7-mediated conversion of the substrate *N*-acetyl-Asp-Glu-Val-Asp-7-amino-4-trifluoromethyl coumarin was monitored fluorometrically (λ_{ex}: 380 nm, λ_{em}: 500 nm).

2.5. Quantification of fibrosis-related gene transcription

Cultures of 5×10^4 CFSC were set up in 25-cm^2 tissue culture flasks using 3 ml standard culture medium and were incubated for 24 h before the medium was replaced by culture medium containing 0.25% FBS. *L. obtusiloba* extracts (135 μg/ml) and/or TGF-β (2 ng/ml) was added after 24 h. In previous studies on CFSC activation kinetics, this treatment regimen resulted in an about 4-fold increase in CI and a more than 10-fold autoinduced TGF-β expression, representing maximum up-regulation. Therefore, these conditions were chosen for all experiments. At different time points, total RNA was directly extracted from cell layers using the RNApure reagent (Peqlab Biotechnologie, Erlangen, Germany). Reversely transcribed complementary DNA templates were amplified by quantitative real-time polymerase chain reaction (PCR; Roche Diagnostics, Mannheim, Germany) using fluorogenic probes labeled with 6-carboxy-fluorescein and 6-carboxy-tetramethylrhodamin (Sigma-Genosys, Steinheim, Germany) and primer pairs (Invitrogen, Karlsruhe, Germany) specific for fibrosis-associated parameters (Table 1). To normalize for differences in cell numbers and variable efficacy of the reverse transcription reactions, we quantified the housekeeping gene glyceraldehyde phosphate dehydrogenase (GAPDH) in parallel reactions.

2.6. MMP activity assays

MMP activities were monitored fluorometrically by cleavage of collagenase-specific substrates [23,24]. All substrates were diluted in MMP activity buffer. The enzymatic activities of MMP-2 and MMP-9 were measured by cleavage of 0.01 mg/ml dye-quenched DQ-gelatine (DQ, Molecular Probes Inc., Eugene, OR). Substrate conversion of human recombinant MMP-2 (Invitek, Berlin, Germany) was monitored by increased fluorescence at 25°C within 2 h. BSA-coated (1 μg/well) wells containing 150 μl substrate solution (DQ-gelatine) received 50 ng activated MMP-2 in 10 μl alone or mixed with 50–200 μg/ml *L. obtusiloba* (final concentration) or the broad-spectrum MMP inhibitor Ilomastat [Millipore (Chemicon), Schwalbach/Ts., Germany].

Cells were treated as described for cell proliferation in 96 wells to assess endogenous gelatinolytic activity of CFSC. After 24 h in the presence of increasing concentrations of *L. obtusiloba* extract, 100 μl of the supernatants was transferred to a black 96-well microtiter plate with clear bottom (Greiner Bio-One). Each well received 50 μl substrate solution (0.01 mg/ml dye-quenched DQ-gelatine) with (67 μM, activation of total gelatinase activity) or without 4-aminophenyl mercuric acetate (APMA) for 60 min at 25°C. Substrate conversion was monitored using a fluorescence microplate reader (SpectraMax, Gemini EM, Molecular Devices).

2.7. Formation of ROS

Intracellular oxidative stress was determined as previously described [25]. Briefly, 1.2×10^5 CFSCs were seeded with standard culture medium into black 96 wells with translucent bottom (Greiner Bio-One) and incubated for 24 h. Cultures received *L. obtusiloba* extract (135 μg/ml) and/or *tert*-butylhydroperoxide (100 μmol/L; Sigma) for 2 h before addition of 5-(6-)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate acetylester (CM-H2DCFDA, 20 μmol/L; Molecular Probes, Karlsruhe, Germany) in basal medium for 1 h. After complete removal of the supernatants and replacement by fresh basal medium, fluorescence (λ_{ex}: 485 nm, λ_{em}: 525 nm) was monitored from the bottom side for 45 min at 37°C.

2.8. Statistics

One-way ANOVAs [All Pairwise Multiple Comparison Procedure (Tukey test)] were performed using SigmaStat for Windows Version 2.03. $P<.001$ was highly significant (**); $P<.05$ was considered significant (*).

3. Results

3.1. L. obtusiloba extract suppresses de novo DNA synthesis in HSC

Effects of *L. obtusiloba* extracts on the proliferation of rat and human HSC were tested after cell cycle synchronization by FBS reduction in the culture medium. To define effective dose ranges with continued serum starvation or with optimum medium composition for exponential growth with 5% FBS, we treated HSCs with increasing doses of *L. obtusiloba* extract up to 700 μg/ml (Fig. 1). *L. obtusiloba* extracts reduced the proliferation of both rat and human HSCs in a dose-dependent manner. With 5% FBS in the culture medium, the reduction patterns were similar and IC_{50} values for the de novo DNA synthesis were about 700 μg/ml of the *L. obtusiloba* extract. Under conditions with continued serum reduction to 0.1% FBS, the IC_{50} for human HSC was 270 μg/ml of *L. obtusiloba* extract. With an IC_{50} below 100 μg/ml of the extract, CFSCs were more sensitive. Since treatment of CFSC with 135 and 270 μg/ml *L. obtusiloba* extract reduced the [^{3}H]thymidine incorporation by 80% and 90%, respectively, these concentrations were used in all subsequent experiments focusing on CFSC.

3.2. L. obtusiloba extract does not affect in vitro morphology and viability

CFSC pretreated with the extract were microscopically examined after sulforhodamine B staining to establish whether the reduction of proliferation as determined by reduced [^{3}H]thymidine incorporation after treatment with *L. obtusiloba* resulted from cytotoxic effects (Fig. 2A). Compared to the nontreated controls, no marked cell death was observed; however, length, diameter and number of cytoplasmic extensions were reduced. Cell numbers at any tested concentration of both the *L. obtusiloba* extract and FBS showed no relevant difference. These alterations were strongest in the treatment group with 0.1% FBS and 270 μg/ml *L. obtusiloba* extract. Sustained viability of CFSC was confirmed by intracellular esterase activity by measuring

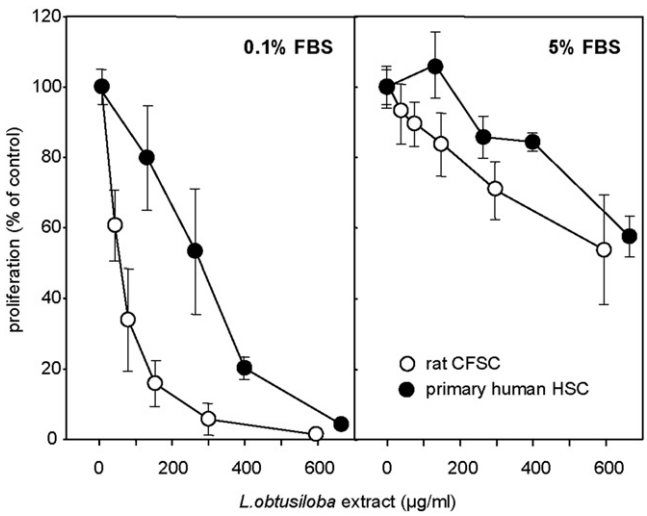

Fig. 1. Proliferation of HSC after treatment with *L. obtusiloba* extract. Cells were incubated in culture medium containing 0.1% or 5% FBS in the presence of different concentrations of *L. obtusiloba* extract as indicated. [^{3}H]Thymidine was added for the last 4 of 16 h and 18 of 30 h to rat CFSC and human HSC, respectively. Proliferation was calculated in comparison to the respective untreated control cells (100%). Results are given as mean values±S.D. of five measurements.

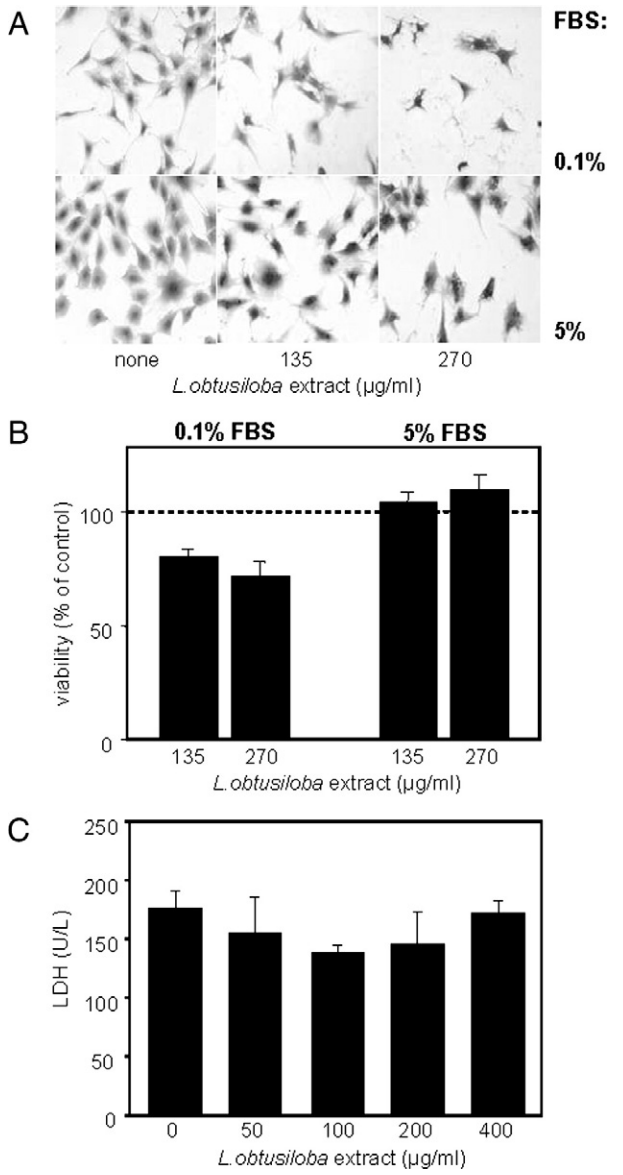

Fig. 2. Morphology and viability of CFSC and cytotoxicity of freshly isolated primary rat hepatocytes in the presence of *L. obtusiloba* extract. Cells were pretreated with *L. obtusiloba* extracts as described in Fig. 1. (A) After 24 h, fixed cell layers were stained with sulforhodamine B and morphology was examined by light microscopy. Original magnification: 40-fold. (B) After 18 h, viability was tested by conversion of 4-methylumbelliferyl heptanoate by intracellular esterases and was calculated in comparison to untreated control cells (100%, dashed line). (C) Primary hepatocytes in 12-well plates after 24 h in medium without FBS were treated with increasing concentrations of *L. obtusiloba* extract for additional 24 h. Cell supernatants were subjected to LDH determination as a measure of cell leakage using a biochemical analyzer. Results are given as mean values±S.D. of five measurements.

4-methylumbelliferyl heptanoate substrate conversion in comparison to control cultures without *L. obtusiloba* extract (Fig. 2B). Under conditions with 0.1% FBS in the culture medium during *L. obtusiloba* treatment, 70–80% of the cells were vital. In the presence of FBS concentrations elevated to 5% in the culture medium, CFSC viability was not affected by the *L. obtusiloba* extract. Thus, we concluded that in the dose range of *L. obtusiloba* extract tested, cytotoxicity as a mediator of reduced CFSC proliferation can be considered negligible. Furthermore, trypan blue dye exclusion (data not shown) and determination of LDH activity in supernatants of *L. obtusiloba*-treated primary rat hepatocytes showed no significant differences to controls (Fig. 2C).

3.3. *L. obtusiloba* extract does not induce apoptosis in HSC and hepatocytes

To assess effects of the *L. obtusiloba* extract on apoptosis, we determined the activity of caspase-3/7 in

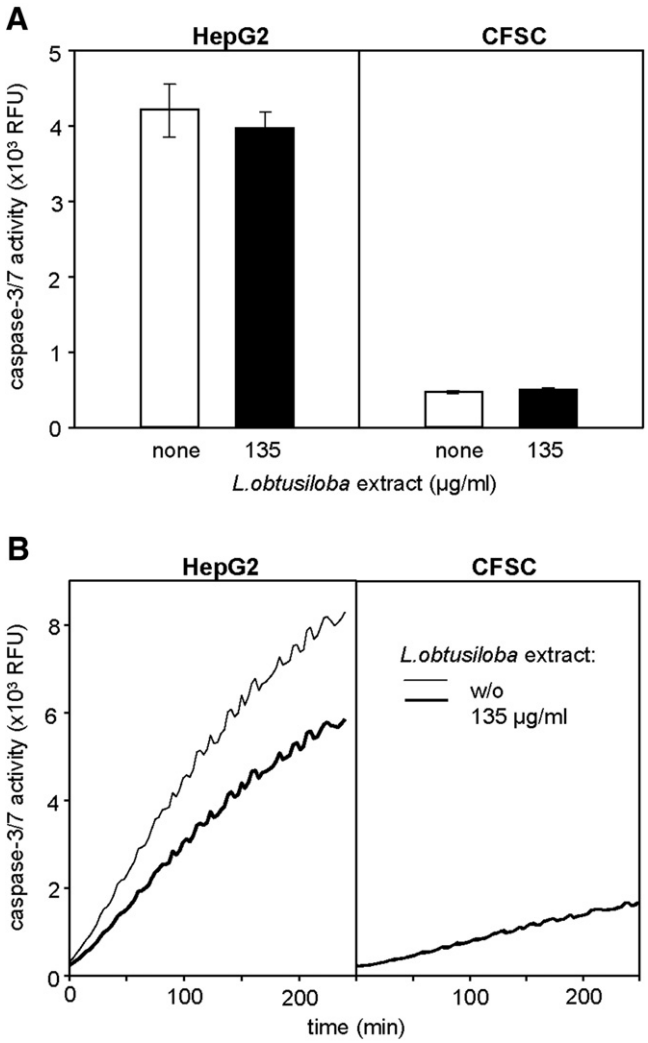

Fig. 3. Effect of *L. obtusiloba* extract on apoptosis of HepG2 and CFSC. Confluent cell layers were treated with 135 µg/well *L. obtusiloba* extract in the absence (A) or presence (B) of 100 nmol/L staurosporine. For HepG2 cells and CFSC, culture medium contained 5% and 0.1% FBS, respectively. After 24 h, caspase-3/7-mediated fluorescent substrate turnover (λ_{ex}: 380 nm, λ_{em}: 500 nm) of the cell lysates was recorded with one data point per minute for 250 min. (A) Caspase-3/7-mediated substrate conversion. Bars represent mean values of individual determination endpoints at 250 min ±S.D. of five measurements. (B) Kinetics of caspase-3/7 activity in the presence of apoptosis-inducing staurosporine as shown for one representative experiment. For CFSC, both curves are superimposed.

liver cells. Since HepG2 cells are routinely used to screen candidate pharmaceuticals for intrinsic hepatotoxicity, they were chosen for reference. *L. obtusiloba* extract (135 μg/ml) alone had no effect on caspase-3/7 activity in HepG2 cells and in CFSC, indicating that the extract did not induce apoptosis (Fig. 3A). Treatment of HepG2 with 100 nmol/L staurosporine and 5% FBS in the culture medium resulted in strongly elevated caspase-3/7 activity after 24 h (Fig. 3B). Addition of 135 μg/ml *L. obtusiloba* extract resulted in caspase-3/7 activity being about 75% lower compared to the control with staurosporine alone (250 min time point). In CFSC under conditions with continued FBS reduction to 0.1% in the culture medium, treatment with staurosporine resulted in enhanced apoptosis after 250 min, which was not further changed by treatment with 135 μg/ml *L. obtusiloba* extract (overlay of curves for *L. obtusiloba*-treated cells and untreated cells).

At concentrations that effectively inhibited HSC proliferation, *L. obtusiloba* extract did not induce apoptosis in HSC but partially prevented staurosporine-induced caspase-3/7 activity in cells representing parenchymal liver cells. Since *L. obtusiloba* extract, in the dose range tested, reduced proliferation of HSC, which was not accompanied by reduced cell viability or increased apoptosis, it was concluded that *L. obtusiloba* extract might induce quiescence in HSC and displays liver protective effects on parenchymal cells.

3.4. L. obtusiloba extract partially abrogates fibrosis-related gene expression in HSC

CFSCs were further activated by addition of 2 ng/ml TGF-β within 24 h as reflected by an increase in alpha smooth muscle actin (α-SMA) expression to assess the effect of the *L. obtusiloba* extract on fibrosis-related gene transcripts (Fig. 4, white columns). Consistent with the onset of a profibrotic gene expression pattern, TGF-β was up-regulated 12-fold. While TIMP-1 and CI expression was also up-regulated three- and twofold, respectively, MMP-3 and MMP-13 were down-regulated. Thus, the reduced expression of matrix-degrading enzymes, the elevated TIMP-1 levels in conjunction with high CI and TGF-β expression mirror a profibrotic gene expression pattern. Adding 135 μg/ml of *L. obtusiloba* extract clearly reversed this pattern (Fig. 4, black columns). The most prominent and significant effect was observed for TIMP-1- and TGF-β-induced cellular TGF-β expression with a reduction of 10% and 30% residual expression, respectively, in comparison to the control without the extract. The observed significant down-regulation of MMP-13 gene expression of CFSC treated with TGF-β and *L. obtusiloba* extract was confirmed in a MMP-13/cat-13 activity assay (data not shown). In line with these findings, *L. obtusiloba* extract significantly elevated expression of MMP-3 by 30%. A trend for reduction in α-SMA expression, indicating HSC activation, and CI, representing the predominant collagen in

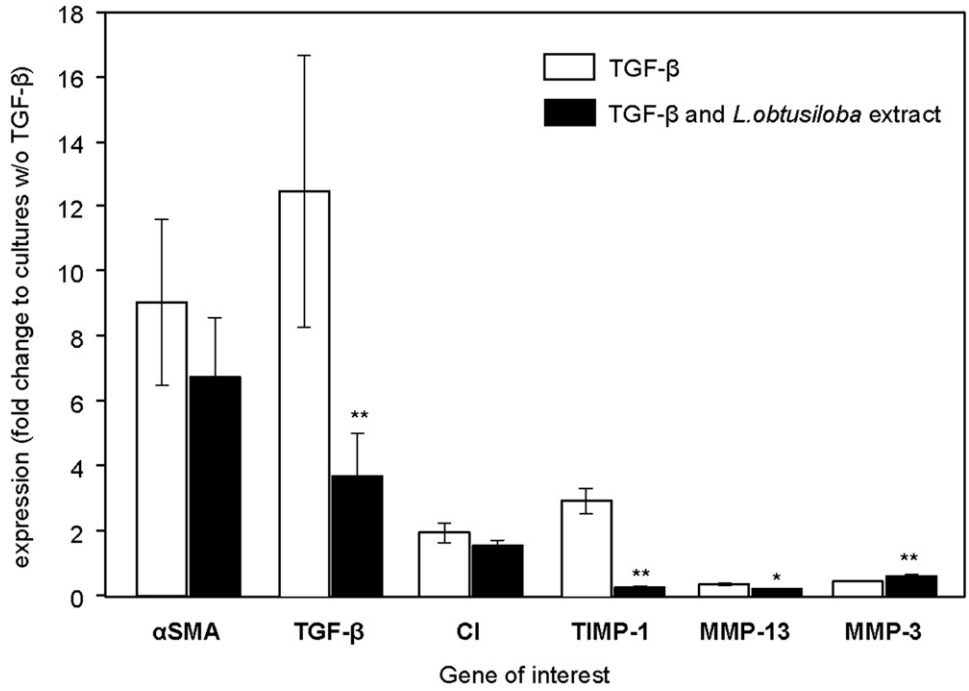

Fig. 4. TGF-β-induced gene expression pattern in CFSC after treatment with *L. obtusiloba* extract. Cell-cycle-synchronized CFSCs were incubated with TGF-β (2 ng/ml) alone or in conjunction with *L. obtusiloba* extract (270 μg/ml) for 24 h or were left untreated for control. The expression of indicated fibrogenesis-associated markers was determined by specific quantitative real-time PCR. Individual transcription levels were normalized to constitutive GAPDH expression, and changes were calculated in relation to the control cultures without TGF-β. Results are given as mean values±S.D. of at least three measurements. One-way ANOVA [All Pairwise Multiple Comparison Procedure (Tukey test)] was performed. $P<.001$ was highly significant (**); $P<.05$ was considered significant (*).

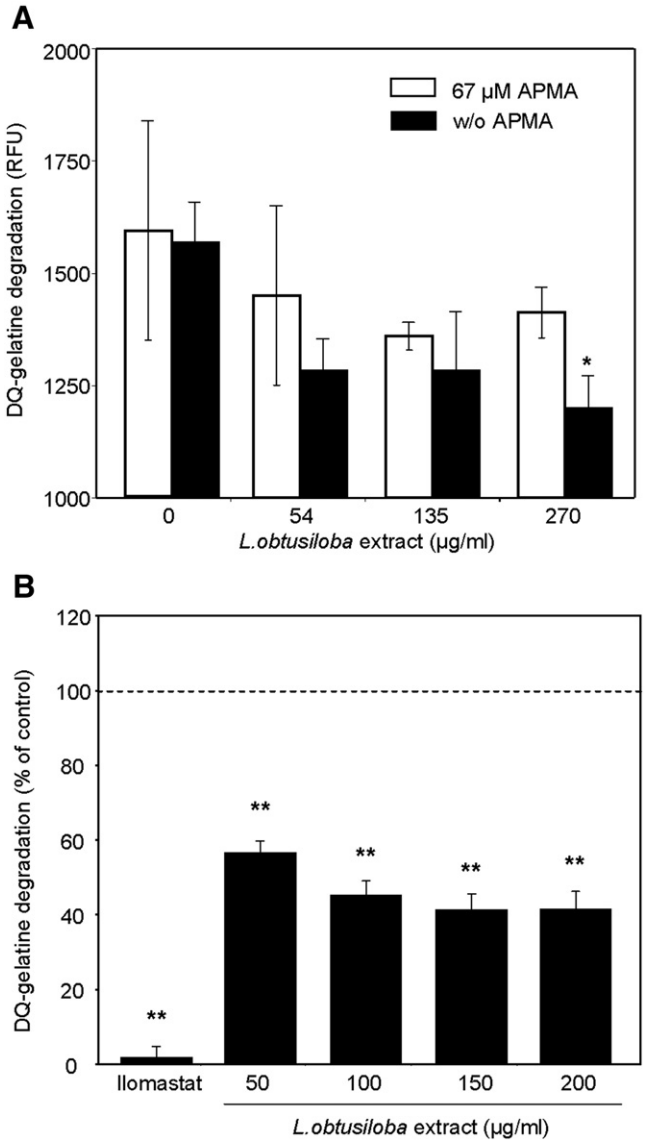

Fig. 5. Effect of *L. obtusiloba* on MMP secretion and activity. (A) Cell-cycle-synchronized CFSCs in 96 wells were treated with increasing concentrations of *L. obtusiloba*, similar to what was described for the proliferation assay. After 24 h incubation, supernatants were mixed with DQ-gelatine substrate in black 96-well plates with clear bottom. Gelatinolytic activity was measured after 20 min at 25°C in a microplate fluorescence reader. In half of the wells, secretion of MMP-2/MMP-9 was determined by measuring total gelatinolytic activity after addition of 67 µM APMA. (B) Activity of human recombinant MMP-2 with (50 to 200 µg/ml) and without (100%) *L. obtusiloba* extract was determined fluorometrically using DQ-gelatine as substrate. As control, MMP-2 activity was blocked by addition of Ilomastat. Results are given as mean values±S.D. of five measurements. One-way ANOVA [All Pairwise Multiple Comparison Procedure (Tukey test)] was performed. $P<001$ was highly significant (**); $P<05$ was considered significant (*).

liver fibrosis, was observed, although it did not reach significance. Taken together, *L. obtusiloba* suppressed a TGF-β-induced profibrotic gene expression pattern, suggesting direct antifibrotic effects of the extract.

3.5. L. obtusiloba reduces gelatine degradation by suppression of MMP-2 activity

To further substantiate the antifibrotic potential of the *L. obtusiloba* extract, the effects on gelatine degradation were investigated. Supernatants of CFSC treated with increasing concentration of the extract with or without APMA were mixed with DQ-gelatine, and gelatinolytic activity was monitored fluorometrically (Fig. 5A). Whereas the secretion of gelatinases was not changed significantly by high concentrations of the *L. obtusiloba* extract (white columns; APMA-treated supernatant correspond to the total endogenous gelatinolytic activity), a significant drop in free gelatinolytic activity of *L. obtusiloba*-treated CFSCs compared to untreated cells was observed (black columns at 270 µg/ml of *L. obtusiloba* extract). In addition, MMP-2, which activates HSC and thus acts profibrotic, was treated with the *L. obtusiloba* extract, resulting in an up to 50% reduction of gelatinolytic activity (Fig. 5B). This points to an additional antifibrotic effect of *L. obtusiloba* via suppression of MMP-2 activity.

3.6. L. obtusiloba neutralizes peroxide-induced oxidative stress response in HSC

In order to elucidate cellular mechanisms of the strong antifibrotic effects observed for CFSC, we investigated the antioxidant properties of the *L. obtusiloba* extract in vital cells. With the use of an assay system that allows monitoring kinetics of the intracellular activation of a

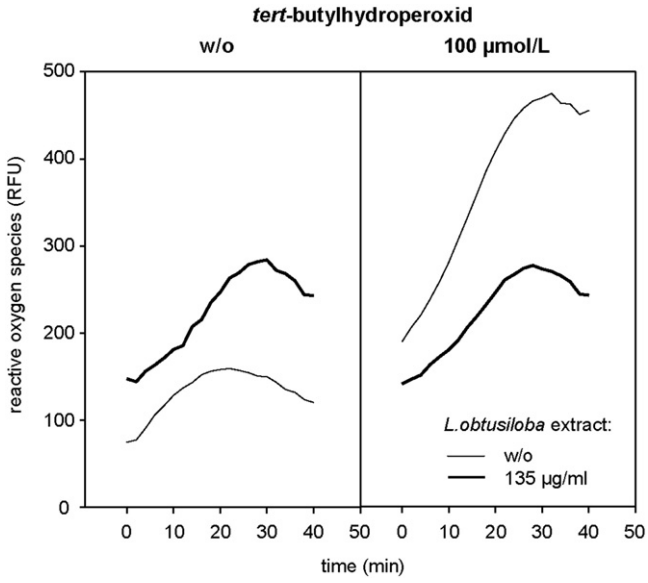

Fig. 6. Effect of *L. obtusiloba* extract on the generation of intracellular oxidative stress in CFSC. Cultures of cell-cycle-synchronized CFSC were treated with 100 µmol/L *tert*-butylhydroperoxide alone or in combination with 135 µg/ml *L. obtusiloba* extract for 2 h. Intracellular activation of CM-H2DCFDA was monitored using black 96-well plates with clear bottom and bottom reading in the cell layer over 45 min with one data point per minute to estimate intracellular oxidative stress. One representative out of three comparable experiments is shown.

fluorescein derivative (CM-H2DCFDA) by ROS and other radicals, 135-μg/ml *L. obtusiloba* extracts were found to induce CM-H2DCFDA activation with a maximum after 30 min of treatment that was about twofold higher compared to CFSC without the extract (Fig. 6, left). Incubation of CFSC with 100 μmol/L *tert*-butylhydroperoxide increased the oxidative stress in the cells up to fivefold compared to untreated cells. Addition of 135 μg/ml *L. obtusiloba* extract abrogated *tert*-butylhydroperoxide-mediated activation of CM-H2DCFDA to levels that were observed for the *L. obtusiloba* extract alone (Fig. 6, right). Thus, the *L. obtusiloba* extract exerts antioxidant properties that may contribute to the antifibrotic potential.

4. Discussion

In the present study, *L. obtusiloba* was tested as part of a large-scale search for active antifibrotic herbals derived from TCM in our laboratory. The shown cell culture data clearly demonstrate that aqueous extracts of *L. obtusiloba* reduced de novo DNA synthesis of activated HSCs, abrogated TGF-β-induced TIMP-1 expression, strongly repressed the typical autocrine loop of TGF-β1 autoinduction [26,27] and reduced the expression of other profibrogenic transcripts. Especially, expression and activity of the collagenase MMP-13 and gelatinase MMP-2 [28] are down-regulated by the *L. obtusiloba* extract, respectively. The role of MMP-13 regarding its expression in HSCs and fibrosis is still in discussion [29]. In our experiments, *L. ob*tusiloba-induced down-regulation of MMP-13 is paralleled by TIMP-1 down-regulation similar to the antifibrotic effects of thalidomide [30], stressing the importance of the MMP/TIMP relation. Importantly, at doses that proved to be antifibrotic, no relevant toxicity to liver parenchymal cells could be observed in vitro. Mechanistically, a potent antioxidant activity seems to be responsible for this effect. To our knowledge, our experimental study is the first to investigate the antifibrotic effects of *L. obtusiloba*. Herbal infusions of *L. obtusiloba* have been traditionally used in Chinese and Korean medicine for treatment of chronic liver diseases and as an anti-inflammatory agent for centuries (K. Kim, personal communication [16]). However, scientific evidence supporting its use is not available through electronic database search except for cell culture studies testing a potential antitumor effect of *L. obtusiloba* extracts in various cancer cell lines. Herein, several lignans and furanolignans were found to be cytotoxic to tumor cells at IC_{50} from 3 to 20 μg/ml [14,15]. However, in these experiments, *L. obtusiloba* was not applied to hepatic parenchymal or mesenchymal cells.

Activated HSCs are the prime source of extracellular matrix produced during fibrosis development; thus, specifically targeting these cells is one of several promising strategies to develop antifibrotic therapies [1]. For example, established experimental fibrosis may recover through HSC apoptosis, as shown by reduced expression of collagen and TIMP in rat liver [31]. Eliminating the cellular source for the neomatrix and for TIMP by programmed cell death results in net matrix degradation [32,33]. Recently, apoptosis was attributed to myofibroblasts derived from portal fibroblasts, while myofibroblasts derived from HSC dedifferentiate [34]. Like baicalein, an active ingredient of Sho-saiko-to [35] and Compound 861 [9], the *L. obtusiloba* extract used in our study reduced HSC proliferation in a wide concentration range without impairing cell viability, suggesting the induction of HSC quiescence. This effect of *L. obtusiloba* compares to the effect of Sho-saiko-to, which was shown to inhibit cellular transformation [36]. In addition, *L. obtusiloba* extract displayed liver protective effects by strongly inhibiting staurosporine-induced apoptosis in parenchymal liver cells paralleled by the absence of cytotoxicity even at very high concentrations.

Once activated, HSCs produce virtually all components of the extracellular matrix, including fibril-forming CI that is up-regulated up to eightfold in advanced fibrosis compared to normal liver [37]. The key profibrogenic mediator TGF-β and its receptors are simultaneously expressed in HSC [38] and lead to a typical autocrine loop of TGF-β1 autoinduction [26,27], making TGF-β a central target for antifibrotic strategies [37,39]. At nontoxic concentrations, the *L. obtusiloba* extract partially abrogated the profibrogenic effect of TGF-β on HSC. Down-regulation of TGF-β as the key cytokine regulating liver fibrosis and TIMP-1 as the main regulator of proteolytic activity in fibrotic and fibrolytic liver [29,40], as well as down-regulation of gelatinolytic (MMP-2) activity, was accompanied by up-regulation of MMP-3 expression. Since comparable effects on matrix turnover were described, for example, for Sho-saiko-to [41], our findings identify aqueous extracts of *L. obtusiloba* as a novel antifibrotic agent. Oxidative stress is involved in the initiation and perpetuation of fibrosis [42,43]. In HSC activation, herbal drugs such as glycyrrhizin and Sho-saiko-to were suggested to reduce oxidative stress in vitro and in vivo, for example, by inhibition of lipid peroxidation or by attenuation of glutathione depletion [8,25,44]. We used CM-H2DCFDA to determine intracellular oxidative stress in CFSC. Measurement of ROS by CM-H2DCFDA should be interpreted cautiously, since CM-H2DCFDA might, by itself, stimulate ROS production [45]. Thus, the high background seen in Fig. 6 without *L. obtusiloba* extract might be due to CM-H2DCFDA-mediated ROS production (thin lines, left side), but stimulation with *tert*-butylperoxide still results in clearly enhanced fluorescence (thin lines, right side), enabling measurement of oxidative stress in CFSC. Our data point to an antioxidative mechanism contributing to the overall effect of *L. obtusiloba*.

Treating chronic liver diseases with botanical drugs has a long tradition, especially in traditional Chinese medicine, but clear evidence for the efficacy of administered compounds is insufficient [44,46]. However, a number of extracts, single compounds and combinations were studied

previously either in cell culture or in vivo and mechanisms were elucidated [36,41,47,48], but convincing clinical data are sparse [44]. While the Western-mechanism-based approach in developing novel treatments is concerned with the search for molecular, cellular and pharmacological bases, Far Eastern medicine relies on experience and empirism and prefers an efficacy-based approach [49,50]. Since neither exists for *L. obtusiloba*, we chose the former. Taken together, we demonstrated clearly antiproliferative, antifibrotic and antioxidative effects of *L. obtusiloba* extracts on HSC and protective effects on parenchymal liver cells. Thus, our results confirm the beneficial potential of *L. obtusiloba* for the treatment of chronic liver diseases. Further studies shall focus on the identification of bioactive molecules from the *L. obtusiloba* extract. In the long run, pharmacokinetics and dynamics and the extension of these findings to in vivo experiments might pave the way for the clinical use of *L. obtusiloba*.

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