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Linalool induces cell cycle arrest and apoptosis in HepG2 cells through oxidative stress generation and modulation of Ras/MAPK and Akt/mTOR pathways

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Abstract

Aims

Linalool is a plant-derived monoterpene with anticancer activity, however its mechanisms of action remain poorly understood. The aim of this work was to elucidate the anticancer mechanisms of action of linalool in hepatocellular carcinoma (HCC) HepG2 cells.

Main methods

Cell viability and proliferation were determined by WST-1 assay and BrdU incorporation, respectively. Cell cycle analysis was assessed through flow cytometry (FC) and western blot (WB). Apoptosis was determined by caspase-3 activity, TUNEL assay and WB. Reactive oxygen species (ROS) and mitochondrial membrane potential (MMP) were analyzed by FC and fluorescence microscopy. Expression of Ras, MAPKs (ERK, JNK and p38) and Akt/mTOR pathways were evaluated by WB.

Key findings

Linalool (0-2.5 mM) dose-dependently inhibited cell proliferation by inducing G0/G1 cell cycle arrest, through Cdk4 and cyclin A downregulation, p21 and p27 upregulation, and apoptosis, characterized by MMP loss, caspase-3 activation, PARP cleavage and DNA fragmentation. Low concentrations of linalool (1.0 mM) reduced membrane-bound Ras and Akt activity whereas higher amounts (2.0 mM) triggered mTOR inhibition and ROS generation, in correlation with MAPKs activation and Akt phosphorylation. ROS scavenger N-acetyl-L-cysteine partially rescued HepG2 cell growth and prevented MPP depolarization, ERK and JNK activation. Moreover, specific ERK and Akt phosphorylation inhibitors potentiated linalool anti-cancer activity, pointing Akt and ERK activation as pro-survival mechanisms in response to higher concentrations of linalool.

Significance

This report reveals that linalool induces G0/G1 arrest and apoptosis in HepG2 cells involving Ras, MAPKs and Akt/mTOR pathways and suggests that linalool is a promising anticancer agent for HCC therapy.

Key words:

Linalool

Hepatocellular carcinoma cells

Cell cycle arrest

Apoptosis

Ras/MAPKs

Akt/mTOR

Reactive oxygen species

1. Introduction

Hepatocellular carcinoma (HCC) is the fifth most frequent cancer worldwide, and the third cause of cancer mortality. Most of actual therapies including surgery, tumor ablation, liver transplantation and chemotherapy are limited to only about 30% of patients, with recurrence rates being very high. For these reasons, HCC 5 years survival after diagnosis reaches less than 5% [1]. In this context, new therapies targeting specific signaling pathways involved in HCC emerge as a promising new tool for HCC treatment.

Phytochemicals arise as potential alternative or supplementary anti-cancer agents mainly due their high effectiveness and low systemic toxicity [2, 3]. Monoterpenes are naturally occurring isoprenoids commonly found in essential oils of citrus fruits and aromatic plants with anticancer properties both *in vitro* and *in vivo* [4,5]. Pioneer reports proposed that monoterpenes acted through inhibition of Ras prenylation, which is essential for its cell membrane anchorage-dependent activity [6,7]. Ras GTPase plays a central role in promoting cell proliferation and survival through its downstream signaling Raf/MEK/ERK and PI3K/Akt/mTOR pathways. Oncogenic Ras exerts its transforming activity in hepatocarcinogenesis mainly by promoting an uncontrolled proliferative signaling mediated by both pathways [8]. For these reasons, several agents capable of blocking Ras and downstream signaling pathways are under evaluation for HCC treatment [9,10].

On the other hand, some phytochemicals could exert their antitumor effect by inducing cellular insults in tumor cells (RE stress, oxidative stress) therefore promoting the activation of stress response pathways including MAPKs (mitogen-activated protein kinases). MAPKs comprise the extracellular signal-related kinases (ERKs), and the stress-activated p38 kinases and c-jun NH2-terminal kinases (JNKs) [11]. Reactive oxygen species (ROS) appear as one of the main mechanisms by which most of anticancer drugs exert their antineoplastic effects, particularly through the activation of MAPKs and the

promotion of mitochondrial membrane depolarization, leading to cell growth arrest, apoptosis, senescence and/or autophagy [12-15].

Linalool (3,7-dimethyl-1,6-octadien-3-ol) is a linear monoterpene alcohol present in essential oils of more than 200 species of plants and herbs like coriander (74.2%), basil (63%), oregano (38%) and grapevine (27.3%), among others [16]. The pharmacological activities of this 10-carbon isoprenoid were evaluated both *in vitro* and *in vivo* and were found having sedative, analgesic, anti-inflammatory, antioxidant, antimicrobial, and antitumor properties among others [17,18]. Linalool was reported to induce cell cycle arrest and/or apoptosis in a wide variety of human cancer cells such as prostate [19], leukemia [20,21], cervical [21], breast [22], sarcoma [23] and epithelial ovarian cancer cells [24], however no reports for HCC cells are available to this respect and the mechanisms by which these effects are produced are not completely understood. We have previously shown the ability of linalool to inhibit the mevalonate pathway and cell growth in HepG2 and A549 cells [25], suggesting that its anticancer activity could be due to the inhibition of Ras prenylation, which is essential to promote tumorigenesis [26].

The purpose of the present study was to examine the molecular mechanisms involved in the antiproliferative activity of linalool in HepG2 hepatocellular carcinoma cells with particular interest in the role of Ras, MAPKs and Akt/mTOR signaling pathways.

2. Materials and Methods

2.1. Chemicals

Linalool>95%, N-acetyl-L-cysteine (NAC), Propidium Iodide (PI), rhodamine-123 (Rh-123), U0126, Ly294002, 2,7-dichlorodihydrofluorescein diacetate (DCFH-DA), apocynin, lipopolysaccharide (LPS) and dimethylsulfoxide (DMSO) were purchased from Sigma Chemical Co. (St Louis, MO, USA). Dulbecco's Minimal Essential Medium (DMEM), fetal bovine serum (FBS), penicillin-streptomycin (P/S) and non-essential amino acids (NEAA) were obtained from Gibco (Invitrogen Corporation, USA). Protease and phosphatase inhibitor cocktails (cat. 88666 and 78420) were from Thermo Scientific (Rockford, USA). Anti-pan-Ras, anti-phospho-ERK (Thr202/Tyr204), anti-phospho-Akt (Ser473), anti-phospho-p38 (Thr180/Tyr182), anti-p38, anti-phospho-JNK (Thr183/Tyr185), anti-phospho-p70S6K (Thr389), and anti-PARP antibodies were from Cell Signaling Technology (Danvers, MA, USA). Anti-p70S6K, anti-Cdk2, anti-Cdk4, anti-cyclin A, anti-cyclin D1, anti-cyclin E, anti-p21, anti-p27, anti-p53 antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-ERK and anti-Akt antibodies were from BD Biosciences. Anti- β -actin antibody was from Sigma Aldrich (Bornem, Belgium).

2.2. Cell culture and treatments

The human hepatocarcinoma cell line HepG2 (HB-8065) was purchased from the American Type Culture Collection. The cells were cultured in T-75 tissue culture flasks in DMEM supplemented with 10% FBS (v/v) plus 1% P/S, 1% NEAA, in 5% CO₂ at 37°C. The medium was changed every 2 days and the cells were passaged when they reached 80% confluence. For experiments, cells were grown under standard conditions and allowed to adhere overnight. Thereafter, they were incubated in DMEM supplemented with

0.1% DMSO (vehicle) or linalool at different concentrations for up to 72 h. The medium was replaced every 24 h. To study the role of ROS in different cellular processes, HepG2 cells were pre-treated with NAC 5 mM or apocynin 250 μ M 2 h prior to co-treatment with linalool.

2.3. Cell viability

HepG2 cells seeded in 96-well plates at a density of 5×10^3 cells per well were incubated in medium supplemented with DMSO 0.1 % or linalool (0-2.5 mM). Cell viability was determined using a colorimetric WST-1 assay (Roche, Vilvoorde, Belgium) according to the manufacturer's instructions.

2.4. Determination of DNA synthesis

DNA synthesis was determined using a colorimetric immunoassay based on bromodeoxyuridine (BrdU) incorporation into the cellular DNA, according to the recommendations of the manufacturer (Roche). The experimental design was identical to the experiments set for the WST-1 assay. DNA synthesis was assessed after 24, 48 and 72 h of treatment and BrdU was added for the last 4 h of the experiment.

2.5. Cell cycle analysis

Cells were plated in 6 well plates at a density of 3×10^5 and allowed to adhere overnight (ON). After treatments, the cells were harvested by trypsinization, centrifuged at $500 \times g$ for 5 min, resuspended in PBS and fixed in 70% ice-cold ethanol for 4 h at 4°C. Then, cells were washed twice with PBS and treated with RNase A (0.1 mg/ml, Sigma Aldrich) for 30 min at 37°C. Finally, cells were stained with DNA staining solution (0.025 mg/ml PI, 0.1% (v/v) Triton X-100) in the dark for 30 min. The stained cells were detected by flow

cytometry using a FACSAriaII flow cytometer (Becton Dickinson, California, USA) and data analyzed by Flow Jo 7.6.2 (Tree Star, Oregon, USA).

2.6. Measurement of caspase-3 activity

Caspase-3 activity was determined using a colorimetric Caspase-3 Assay Kit (Sigma, St. Louis, MO, USA) according to the manufacturer's instructions.

2.7. TUNEL Assay

Apoptotic DNA fragmentation was assessed using a TUNEL assay (In-Situ Cell Death Detection Kit, TMR Red, Roche, Mannheim, Germany). HepG2 cells were plated on sterile coverslips (24x24 mm) in 6 well plates at a density of 3×10^5 and allowed to attach overnight. After treatment with linalool (1.0-2.0 mM) for 24 and 48 h, cells were washed three times with PBS and then fixed with 4% paraformaldehyde in PBS for 60 min at room temperature. The cells were then washed twice and permeabilized with 0.1% Triton X-100 (v/v) in PBS for 2 min on ice followed by TUNEL reaction mixture for 1 h at 37°C in the dark. After the reaction, the slides were mounted with ProLong® Gold Antifade Reagent with DAPI (4',6-diamidino-2-phenylindole dihydrochloride) for nuclear staining (Life Technologies, Carlsbad, CA) and examined under an Olympus BX51 Fluorescence Microscope (Tokyo, Japan) equipped with an Olympus DP70 digital camera. Images were analyzed using ImagePro Plus v. 5.1 software (Media Cybernetics, Silver Spring, MD).

2.8. Measurement of intracellular ROS

Intracellular ROS production was quantified using the peroxide-sensitive fluorescent probe DCFH-DA (Sigma Aldrich). HepG2 cells were seeded in 6-well plates (3×10^5) and treated for 3h with DMSO 0.1 % (Control), linalool (1.0, 1.5 and 2.0 mM), hydrogen peroxide 0.4

mM (positive control), or pretreated with NAC 5 mM 2 h prior to addition of linalool 1.5 mM. Then, cells were stained in serum-free DMEM containing 10 μ M DCFH-DA in the dark for 30 min at 37 °C. Thereafter, cells were harvested, washed twice with PBS, resuspended in 1 ml PBS and immediately examined by flow cytometry using a FACSCantoII flow cytometer (Becton Dickinson, California, USA) with an excitation wavelength of 488 nm and emission wavelength of 525 nm. Data were analyzed by Flow Jo 7.6.2 (Tree Star, Oregon, USA). In a parallel experiment, after treatment of HepG2 cells seeded in 24-well plates (5×10^4), cells were washed twice with PBS and exposed to 10 μ M DCFH-DA in the dark for 30 min at 37 °C. After washed twice with PBS, cells were imaged under an Olympus LX71 Inverted Fluorescence Microscope (Tokyo, Japan) equipped with an Olympus digital camera (E-330).

2.9. Measurement of Mitochondrial Membrane Potential (MMP)

MMP was determined using fluorescent dye Rh-123, a membrane-permeable cationic fluorescent dye which accumulates in mitochondria as a direct function of the membrane potential. HepG2 cells were treated with linalool 1.5 mM for 3, 24 and 48 h and then collected and washed twice with PBS. The cell suspension was stained with 5 μ M Rh-123 for 30 min at 37°C in the dark, centrifuged at 500 x g for 7 min, washed twice with PBS and resuspended in 1 ml PBS. The fluorescence intensity was measured immediately by FACSCantoII flow cytometer with an excitation wavelength of 488 nm and emission wavelength of 525 nm. In addition, HepG2 cells seeded in 24-well plates (5×10^4) were incubated with linalool 1.5 mM for 3, 24 and 48 h, linalool 1.0 to 2.0 mM for 24h, or rotenone 5 μ M for 12 h as a positive control. After washed three times with PBS, cells were stained with 10 μ M Rh-123 in free-serum DMEM for 30 min at 37°C in the dark, again

washed three times with PBS and imaged under an Olympus LX71 Inverted Fluorescence Microscope (Tokyo, Japan) equipped with an Olympus digital camera (E-330).

2.10. Western Blot analysis

Cells were plated in 100 mm Petri dishes at a density of 2×10^6 and allowed to adhere ON. After treated with linalool 1.0 to 2.0 mM for 24-48 h, cells were washed twice in PBS and lysed in RIPA buffer [(50 mM Tris-HCl-pH 8.0, 150 mM NaCl, 1.0 % (v/v) NP-40, 0.5% (w/v) sodium deoxycholate, 0.1% SDS, and 1 mM EDTA)] containing protease and phosphatase inhibitors for 30 minutes on ice and centrifuged 15 min at $14,000 \times g$ at 4°C . The concentration of total proteins was measured BCA protein assay (Pierce, Rockford, IL). Equal amounts of protein (30-60 μg) were resolved on a 10-12% SDS-PAGE and transferred onto a polyvinylidene fluoride membranes (PVDF, Amersham, GE Healthcare). Membranes were blocked for 60 min in 5% bovine serum albumin (BSA) in TBST buffer (150 mM NaCl, 10 mM Tris-HCl- pH 7.4, 0.05% Tween-20) at room temperature and incubated with primary antibodies at a dilution of 1:200 to 1:1000 at 4°C , overnight. Immunoreactivity was detected by using horseradish peroxidase-conjugated anti-rabbit or anti-mouse IgG and visualized with the Western Lightning Chemiluminescent Reagent Plus (Perkin Elmer, Boston, MA) by the Gel Doc TMXR System 170–8170 device and software (Bio-Rad, Nazareth, Belgium). β -actin was used as loading control.

2.11. Ras subcellular localization

The different subcellular fractions were obtained using a protocol adapted from Palozza et al.[27]. Briefly, cells were washed twice with PBS and lysed in lysis buffer (10 mM Tris-HCl-pH 7.4, 10 mM NaCl, 3 mM MgCl_2 , 0.5% TritonX-100) supplemented with protease inhibitors for 15 min on ice. Thereafter, cells were collected and homogenized by 20

passes through a 27 ga needle on ice. The homogenate was further centrifuged for 10 min at 500 x g to obtain the postnuclear fraction, which was ultracentrifuged at 100,000 x g for 60 min (Beckman Optima LE-80K, SW60Ti rotor) to obtain a pellet of the cell membrane fraction, and the cytosol fraction in the supernatant. The pellet was suspended in membrane resuspension buffer [10 mM Tris-HCl-pH7.4, 100 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% TritonX-100, 0,1% SDS] supplemented with protease inhibitors. Protein samples from membrane (100 µg), cytosol and homogenate (20 µg each one) were subjected to Western Blot analysis as described earlier (section 2.10).

2.12. Statistics

Results are expressed as relative change compared with DMSO controls and presented as means \pm SD. Statistical significance was determined using the Student t-test. Significance was set at $p < 0.05$ (GraphPad inStat program).

3. Results

3.1. Linalool suppressed HepG2 cells proliferation and growth

To investigate linalool-induced growth inhibition, we performed a WST-1 assay to analyze HepG2 cell viability. Significant cell viability inhibition was observed for concentrations from 1.0 mM after 24-48 h, and from 0.5 mM after 72 h, then cell viability decreased in a dose- and time- dependent manner (Fig. 1A). Given that WST-1 assay evaluates healthy cells but does not distinguish between quiescent or actively dividing cells, we also used the BrdU incorporation assay to measure specific cell proliferation rate. Linalool impaired DNA synthesis in HepG2 cells in a significant dose-dependent manner already at 0.5 mM at 24 h and at 0.25 mM after 48-72 h (Fig. 1B). These results suggest that linalool acts as an antiproliferative drug at concentrations where cell viability is not altered.

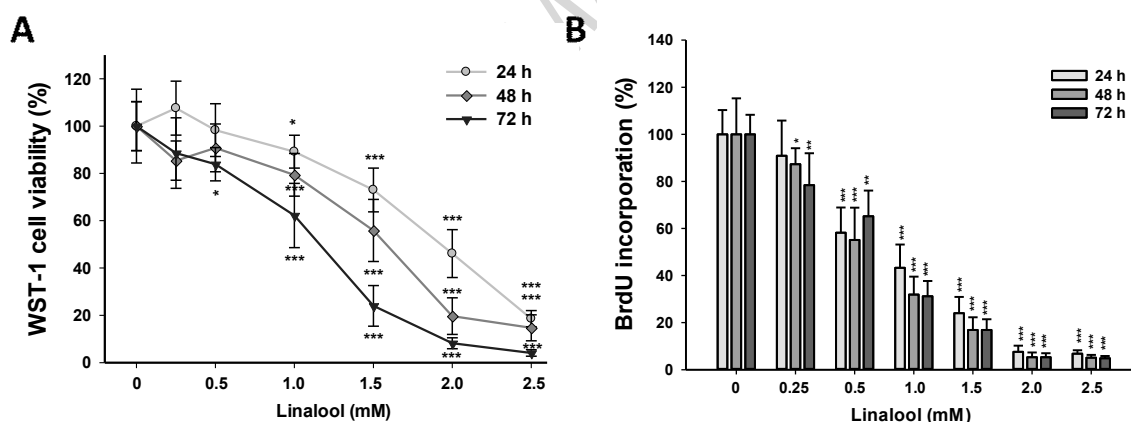


Fig. 1. Linalool inhibited HepG2 cell viability and proliferation. Cells were plated in 96-wells plates and incubated with various concentrations of linalool (0-2.5 mM) for 24, 48 and 72 h. (A) Cell viability assessed by WST-1 expression showing a dose- and time-dependent decrease in HepG2 cell viability. (B) BrdU incorporation into DNA showing a dose-dependent inhibition of HepG2 cell proliferation exerted by linalool. Data are presented as the mean \pm SD (n=8). *p<0.05; **p<0.01; ***p<0.001.

3.2. Linalool promoted G0/G1 phase cell cycle arrest

In order to determine the potential mechanisms by which linalool impairs HepG2 cell proliferation, cell cycle progression was analyzed by flow cytometry (Fig. 2A,B). As shown in Fig. 2A, after 24 h exposure of HepG2 cells to 1.0mM linalool, a significant G0/G1 phase arrest was observed which further increases with 2.0mM of linalool. In parallel, a concomitant decrease of cells in S phase from 20.7% in control cells to 6.2% for 2.0 mM linalool ($p<0.001$) occurred whereas no significant differences were found in the G2/M population. Similar results were observed for 48 h of treatment, with however a higher percentage of cells arrested in G0/G1 (79.2%) after incubation with linalool 1.0 mM with respect to 24 h treatment ($p<0.05$, Fig. 2B).

To get some insight into the molecular mechanisms of linalool-induced G0/G1 arrest, expression of cyclins A, D1 and E, Cdk2, Cdk4, p21, p27 and p53 were analyzed by Western Blotting after 24 h treatments (Fig. 2C,D). Although, linalool induced no significant changes in Cdk2 and cyclin D1 levels, a dose-dependent down-regulation in the expression of Cdk4 and cyclin A up to 53 and 45%, respectively ($p<0.05$) was observed. Moreover, a slightly but significant 17% decrease in cyclin E levels was also found in HepG2 cells incubated with linalool 2.0 mM (Fig. 2D). Expression of both p21 and p27, negative regulators of G1 progression, strongly increased after 1.0 and 1.5 mM linalool treatments (1.4-1.8 folds, $p<0.05$), but not in cells exposed to linalool 2.0 mM with respect to control cells. p53 levels notably diminished in HepG2 cells in response to linalool treatments starting from 1.5 mM onwards ($p<0.05$).

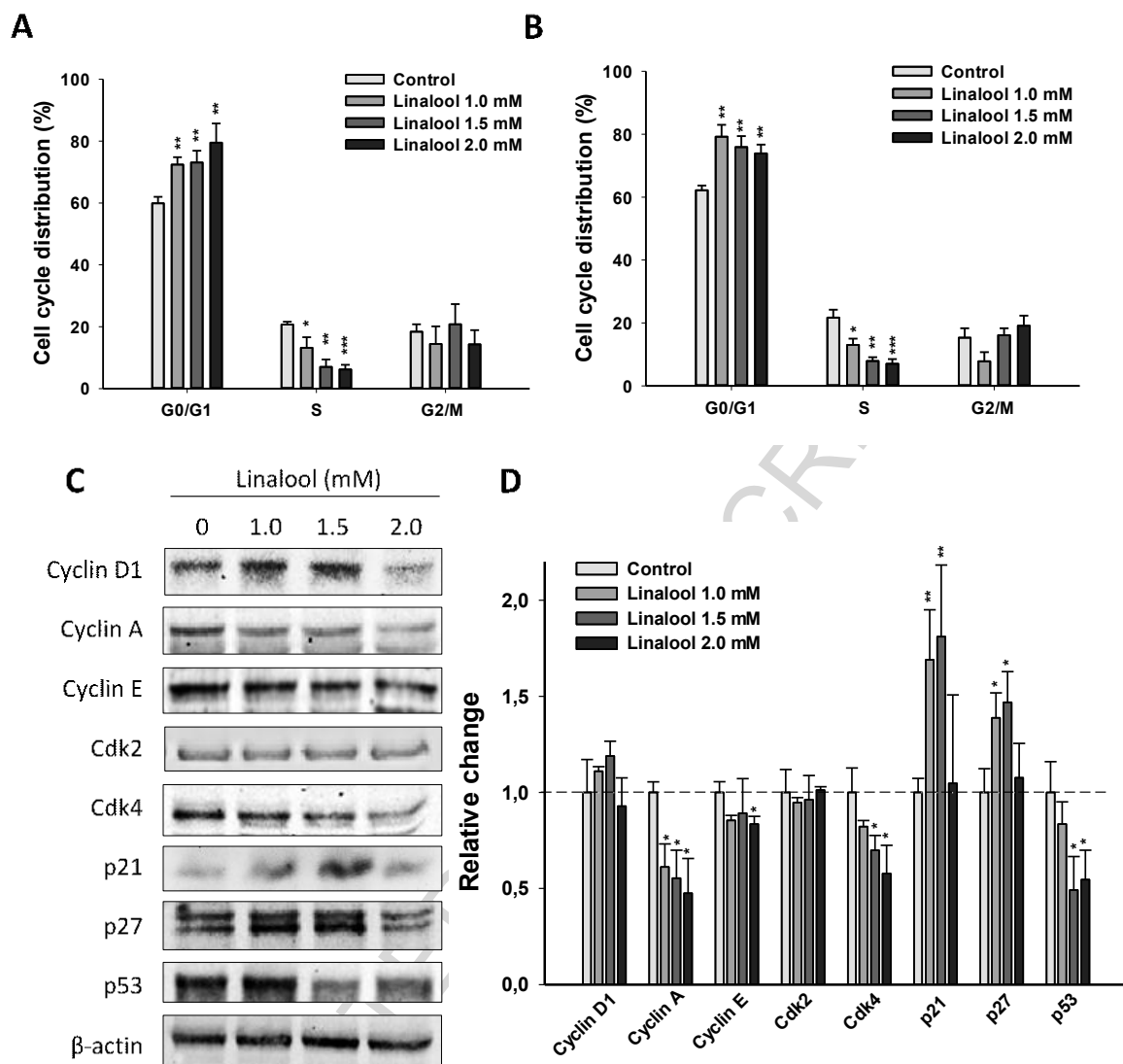


Fig. 2. Linalool induced G0/G1 cell cycle arrest. (A-B) Cell cycle distribution in HepG2 cells after exposed to linalool for 24 h (A) and 48 h (B). Data are expressed as mean \pm SD (n=4). (C) HepG2 cells were incubated with increasing concentrations of linalool for 24 h. Representative immunoblots of cyclin A, cyclin D1, cyclin E, Cdk2, Cdk4, p21, p27, p53 and β -actin (loading control). (D) Quantification of protein levels by densitometric analysis (n=4). Data are expressed as the mean \pm SD. *p<0.05; **p<0.01; ***p<0.001

3.3. Linalool induced apoptosis in HepG2 cells

We next assessed the ability of linalool to induce apoptosis in HepG2 cells. We first assayed caspase-3 activity, an effector caspase which is activated upon initiation of both the intrinsic and extrinsic apoptotic pathways. No caspase-3 activation was detected after 24 h treatments in response to any of the concentrations tested (1.0-2.0 mM) whereas a significant 2.2 to 2.4-fold induction was observed after exposition to linalool 1.5 and 2.0 mM for 48 h (Fig. 3A). Another hallmark for apoptotic cell death is the DNA fragmentation produced by caspase-activated endonucleases. Therefore, DNA breaks in cells were analyzed by means of the TUNEL assay (Fig. 3B). No significant differences were found after 24 h linalool treatments respect to control cells (data not shown) whereas TUNEL-positive apoptotic nuclei increased from 0.32% to 5.77% and 10.04% in HepG2 cells treated for 48 h with linalool 1.5 and 2.0 mM, respectively (Fig. 3C, $p < 0.001$). Furthermore, a third line of evidence for linalool-induced apoptosis was obtained through the analysis of the apoptotic marker PARP, which is cleaved after caspase-3 activation. A significant dose-dependent decrease in the PARP levels in parallel with the increase of the cleavage product was detected after 48 h linalool treatment (Fig. 3D). Altogether, these results demonstrates that linalool is able to induce apoptosis in HepG2 cells after 48 h treatments at concentrations starting from 1.5 mM.

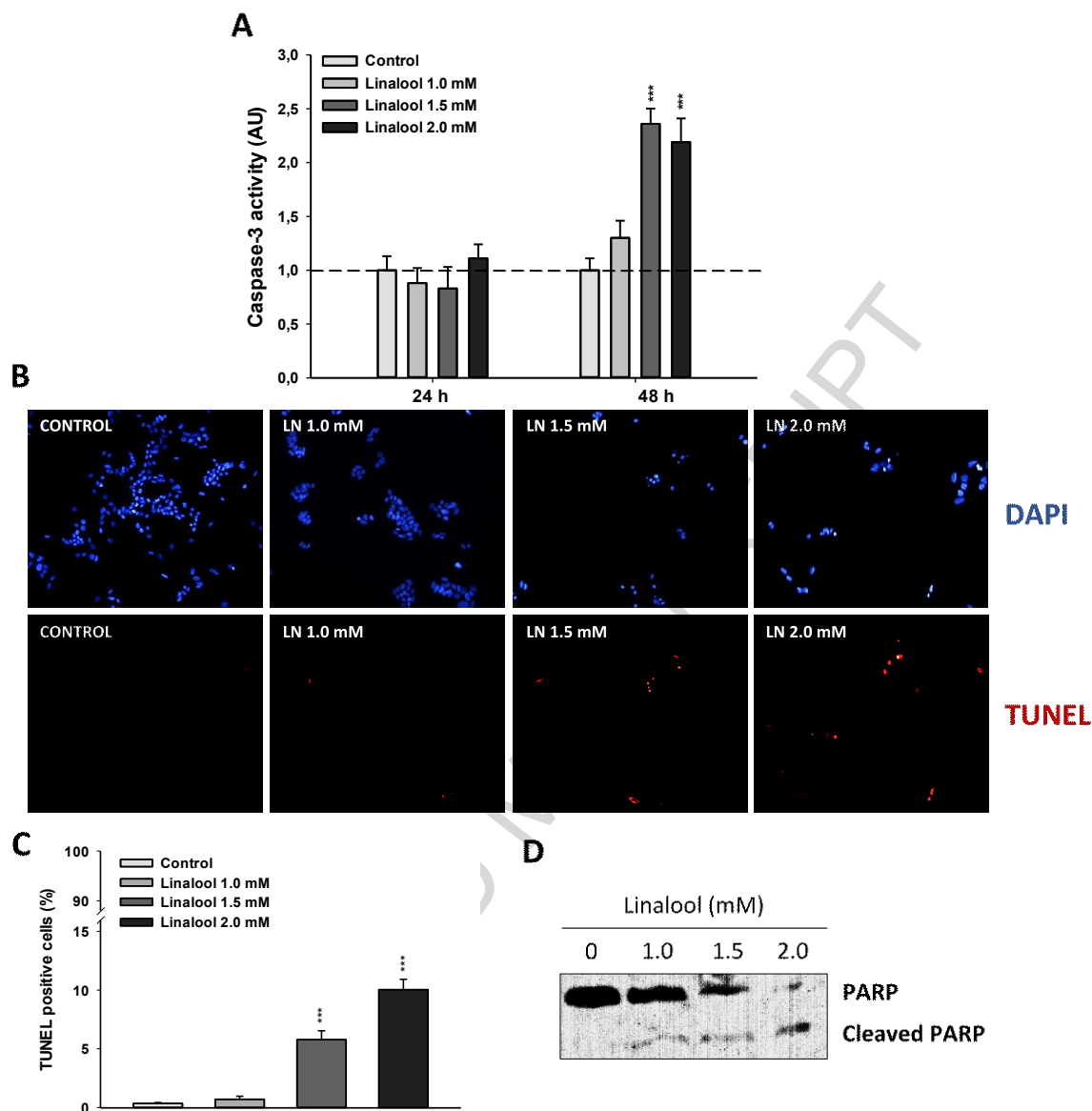


Fig. 3. Linalool induced apoptosis in HepG2 cells. HepG2 cells were treated with linalool (1.0, 1.5 and 2.0 mM) for 24 and 48 h and apoptosis was determined by different methodologies. (A) Caspase-3 activity was determined colorimetrically and the results were normalized to control cells (AU= arbitrary units, n=4). Data are presented as the mean \pm SD; * p <0.05; ** p <0.01; *** p <0.001. (B) Representative fluorescence micrographs of apoptotic cells labeled by TUNEL assay after 48 h treatments with DMSO 0.1 % (Control) or linalool (LN) at the mentioned concentrations. Cells were examined under an Olympus BX51 fluorescence microscope (magnification \times 10). Upper panel, nuclei stained with DAPI; lower panel, red-stained TUNEL positive cells. (C) Quantification of

TUNEL positive cells. About 10-15 fields (100-200 cells/field) of three independent experiments were evaluated. Data are expressed as the mean \pm SD. * $p<0.05$; ** $p<0.01$; *** $p<0.001$. (D) Representative immunoblot showing the poly ADP ribose polymerase (PARP) cleavage (apoptotic marker) after 48 h treatment (n=4).

3.4. Linalool effects on Ras expression

We next measured the levels of Ras on the cell membrane and in cytosolic fractions after exposing HepG2 cells to linalool 1.0, 1.5 and 2.0 mM for 24 and 48 h (Fig. 4A-B). After 24 h, Ras membrane expression decreased about 30-35% for all tested concentrations ($p<0.001$) without significant changes in cytosolic Ras levels (Fig. 4C). The effect was even more pronounced after 48 h, where membrane Ras strongly decreased up to 50% ($p<0.001$). In parallel, a modest increase of 16% and 25% ($p<0.05$) in cytosolic Ras and total Ras, respectively, was observed upon treatment with linalool 1.5 mM (Fig. 4D).

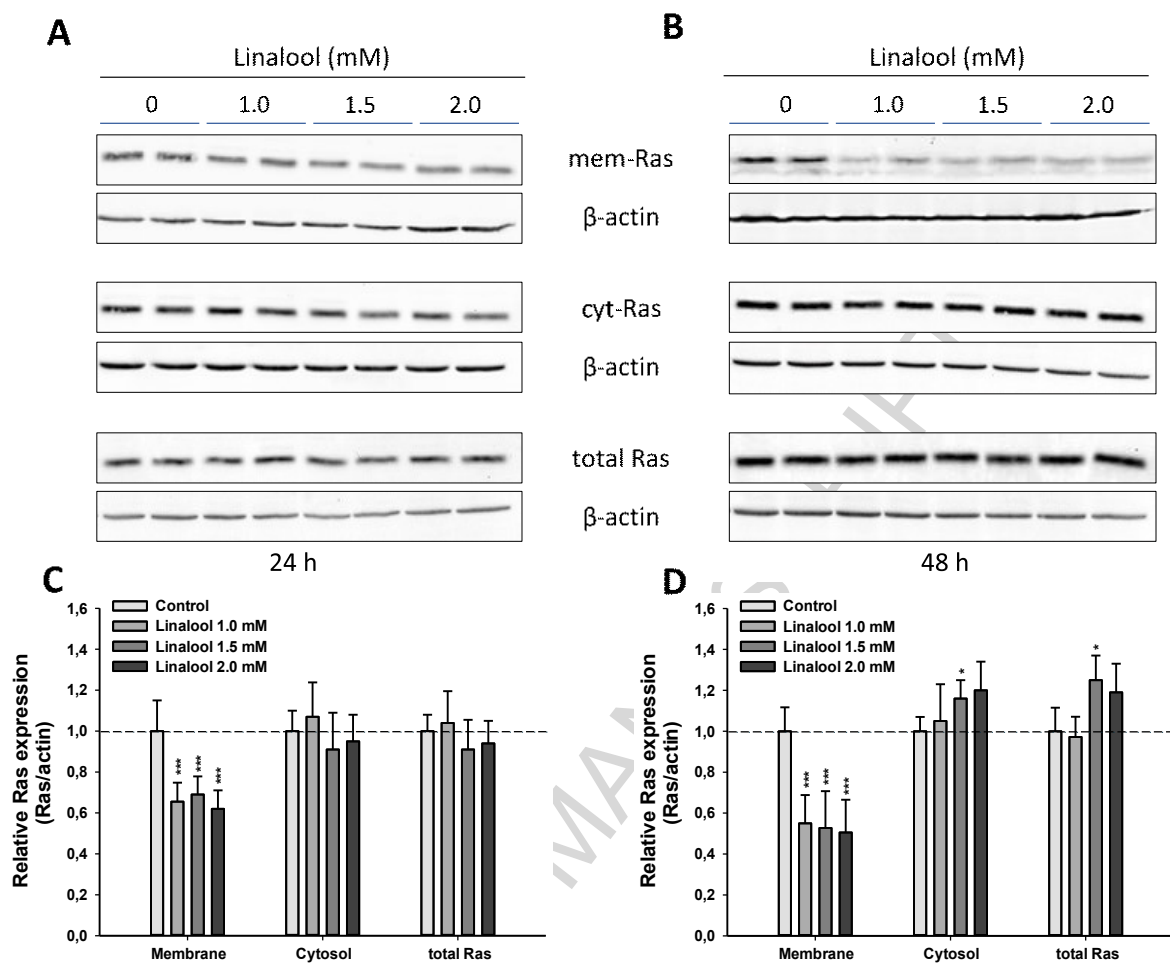


Fig. 4. Effect of linalool on Ras expression. HepG2 cells were treated with linalool (1.0, 1.5 and 2.0 mM) for 24 and 48 h. (A-B) Representative Western Blots of Ras protein in the membrane (mem-Ras) and cytosolic (cyt-Ras) fractions and total Ras after 24 h (A) and 48 h (B) treatments. (C-D) The resulting bands for 24 h (C) and 48 h (D) treatments were quantified by densitometric analysis and then normalized to their respective β -actin. Data are expressed as relative change compared to the control group, arbitrarily set at 1 and expressed as the mean \pm SD of two independent experiments performed by quadruplicate. * p <0.05; ** p <0.01; *** p <0.001.

3.5. Linalool induced ROS production

We next evaluated whether linalool was able to induce ROS generation by flow cytometry and fluorescence microscopy (Fig. 5A). Linalool substantially induced ROS generation at 1.5 and 2.0 mM (Fig. 5A, lower panel). In addition, flow cytometry analysis also showed that linalool significantly increased ROS levels at 1.5 mM (3.2 folds) and 2.0 mM (6.8 folds) and pre-treatment with the antioxidant NAC was able to prevent such an increase (Fig. 5B). To get some insight into the source of ROS generation, the effect of apocynin (a specific NADPH oxidase inhibitor) on linalool-induced ROS production was analyzed under fluorescence microscopy. As shown in figure 5C, apocynin did not prevent linalool-induced ROS production.

In order to analyze the involvement of ROS in linalool-induced cell growth inhibition, HepG2 cells were treated with linalool in absence or presence of 5 mM NAC or 250 μ M apocynin. After 24 h treatments with linalool 1.5 and 2.0 mM, NAC was able to restore cell viability from 73.5 and 55.3% to 83.8 and 80.2% respectively. However, after 48 and 72 h, this ROS scavenger molecule could only partially rescue cell growth. On the other hand, apocynin was unable to prevent cell viability loss induced by linalool at any of the tested conditions (Fig. 5D).

These results suggest that ROS production, at least in part, contributes to the antiproliferative effects exerted by linalool.

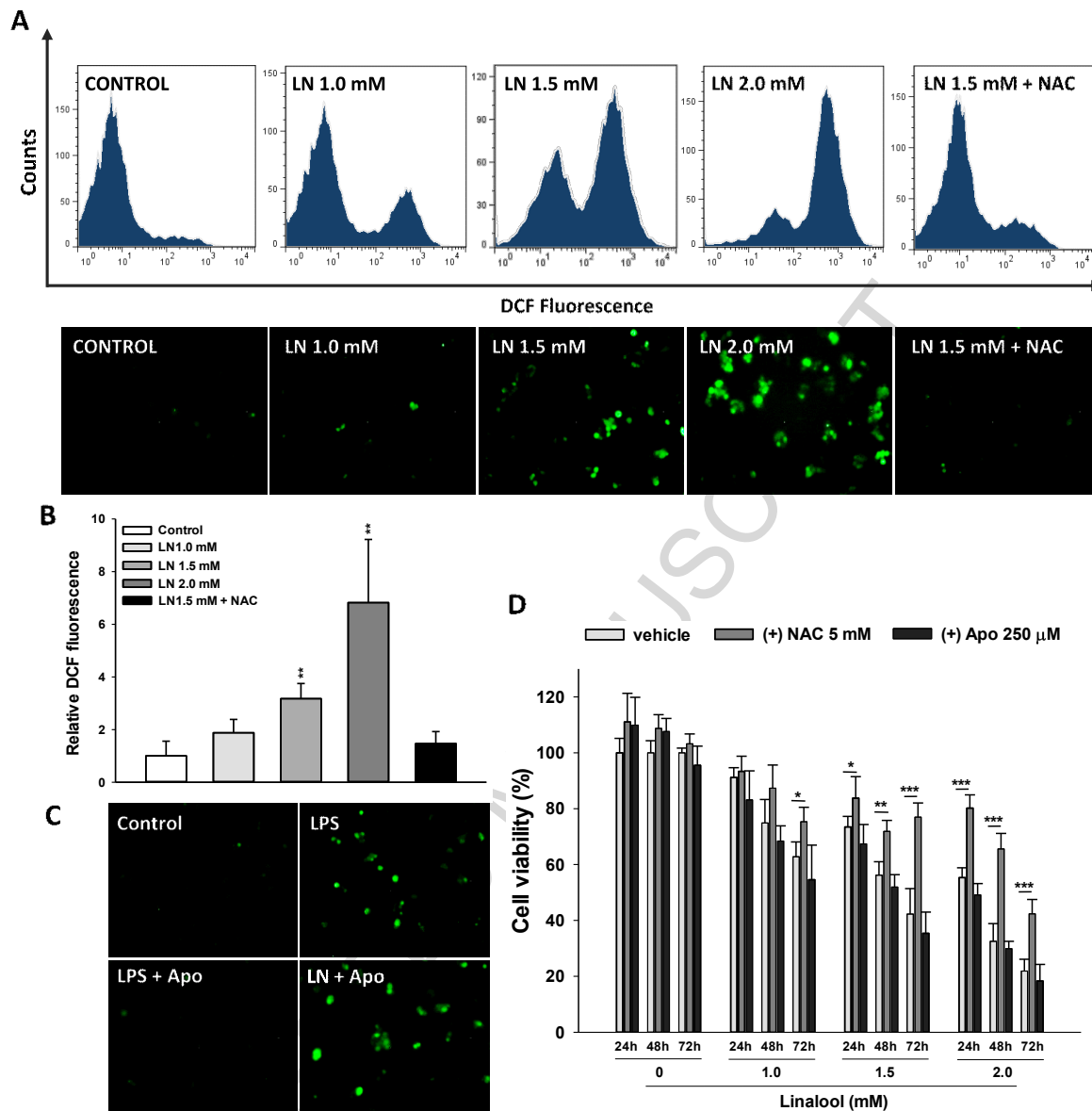


Fig. 5. Oxidative stress induced by linalool. (A-B) HepG2 cells were treated with DMSO 0.1 % (Control), linalool (LN, 1.0, 1.5 and 2.0 mM) or pretreated with NAC 5 mM 2 h prior to addition of linalool 1.5 mM (LN 1.5 mM + NAC) for 3h, and then stained with 10 μ M DCFH-DA for 30 min. (A) ROS production was evaluated by flow cytometry (upper panel). Representative images of HepG2 cells showing ROS positive cells under a fluorescence microscope (20X, lower panel). (B) The fluorescence intensity of the cells analyzed by flow cytometry was calculated relative to that of control (set at 1). Data are expressed as the means \pm SD of three independent experiments (n=4). *p<0.05; **p<0.01; ***p<0.001. (C) HepG2 cells were treated with DMSO 0.1 % (Control), LPS (1

µg/ml) or pretreated with apocynin (Apo, 250 µM) 2 h prior to addition of LPS (LPS + Apo) or LN 1.5 mM (LN + Apo), and intracellular ROS were detected under a fluorescence microscope (20X). (D) HepG2 cells were pre-incubated with the ROS scavenger NAC (5 mM) or apocynin (250µM) 2 h prior to the addition of linalool (1.0 – 2.0 mM) for 24, 48 and 72 h. Cell viability was assessed by WST-1 expression. Data are presented as mean ± SD (n=8). *p<0.05; **p<0.01; ***p<0.001.

3.6. Linalool decreased Mitochondrial Membrane Potential (MMP)

MMP collapse is a sign of ROS-induced mitochondrial damage and a major stimulus for apoptosis. Linalool 1.5 mM produced a time-dependent loss in MMP after 24 h (p<0.05) and 48 h (p<0.01) of treatment, as evidenced by flow cytometry (Fig. 6A). To further confirm these findings, HepG2 cells were exposed to linalool in time-course and dose-dependent manner and MMP evaluated under fluorescence microscopy (Fig. 6). A time- and dose-dependent decrease in rh-123 content in HepG2 cells (green fluorescence) was observed, supporting the ability of linalool to alter mitochondrial integrity. Furthermore, NAC prevented cell depolarization, as shown by a greater green fluorescence intensity in HepG2 cells pre-treated with the antioxidant agent before addition of linalool 1.5 mM for 48 h (Fig. 6B, right upper panel).

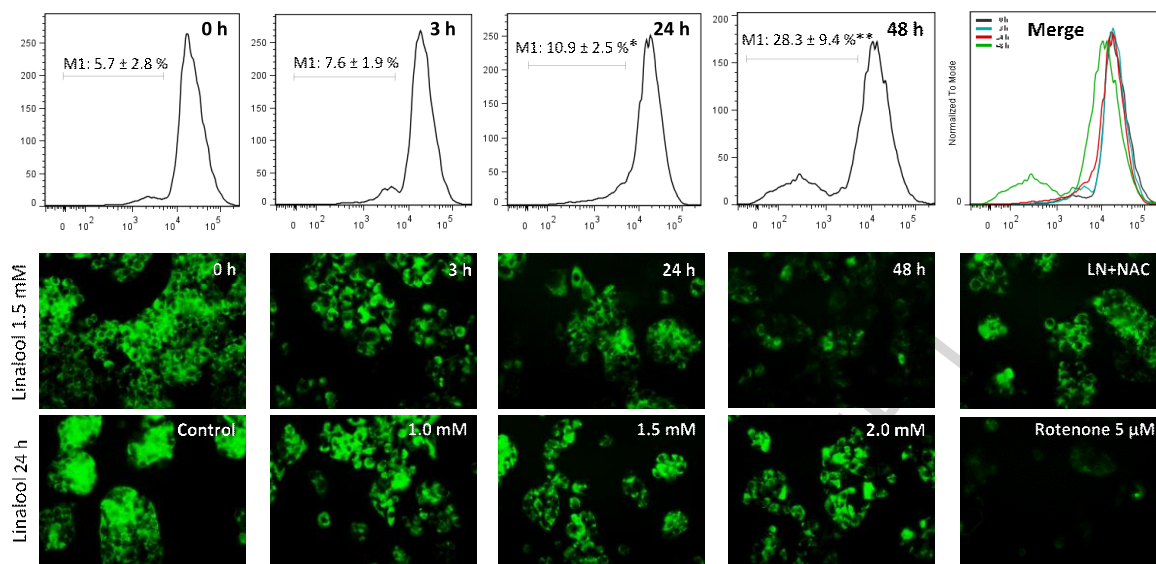


Fig. 6. Effect of linalool on mitochondrial membrane potential (MMP) in HepG2 cells. (A) HepG2 cells were treated with DMSO 0.1 % (Control) or linalool 1.5 mM at different time points (0-48 h) and MMP was determined by flow cytometry. M1 gates show the percentage MMP loss. Data are expressed as the means \pm SD of two independent experiments (n=4). *p<0.05; **p<0.01; ***p<0.001. (B) Representative images of HepG2 cells showing loss of MMP under a fluorescence microscope (40X). Upper panel: cells were treated with DMSO 0.1 % (Control), linalool 1.5 mM at different time points (0-48 h) or pre-incubated with the ROS scavenger NAC (5 mM) 2 h prior to the addition of linalool 1.5 mM. Lower panel: cells were treated for 24 h with DMSO 0.1 % (Control), linalool at different concentrations (1.0 – 2.0 mM) or rotenone 5 μ M (12 h, positive control).

3.7. Linalool induced MAPKs activation

Based on the finding that linalool affected Ras expression and promoted ROS accumulation, the three stress-activated MAPK kinases (ERK, JNK, p38) were analyzed. HepG2 cells were treated with linalool 1.0, 1.5 and 2.0 mM for 24 h and protein expression and phosphorylation were evaluated by Western Blot (Fig.7A). Linalool significantly induced the phosphorylation of the three MAPKs starting from 1.5 mM whereas total

protein levels remained unchanged in response to linalool treatments. ERK, JNK and p38 phosphorylation increased 39 and 42%, 38 and 49%, and 265 and 308% after treated with linalool 1.5 and 2.0 mM respectively ($p < 0.05$ in all cases). To determine whether ROS production was responsible for MAPKs activation, HepG2 cells were treated with linalool 1.5 mM for 24 h in absence or presence of 5 mM NAC (added 2 h before). As shown in Fig. 7B, NAC was able to at least partially prevent JNK and ERK phosphorylation ($p < 0.05$), whereas no significant changes were observed for p38 phosphorylation.

In contrary to its pro-survival function, sustained ERK activation is able to induce cell death under some circumstances [12]. In order to explore the role of ERK activation in linalool-exposed HepG2 cells, ERK phosphorylation inhibitor (U0126, 10 μ M) was added to the culture medium 2h before the addition of linalool (1.0-2.0 mM) for additional 24 h. As expected, cell viability was reduced in HepG2 cells cultured with 10 μ M U0126 alone, whereas in combined treatments with linalool 1.5 and 2.0 mM (concentrations where ERK activity is increased), ERK inhibition induced a further strong decrease in cell viability to 33 and 8%, respectively, suggesting a pro-survival role for ERK activation in this context (Fig. 7C).

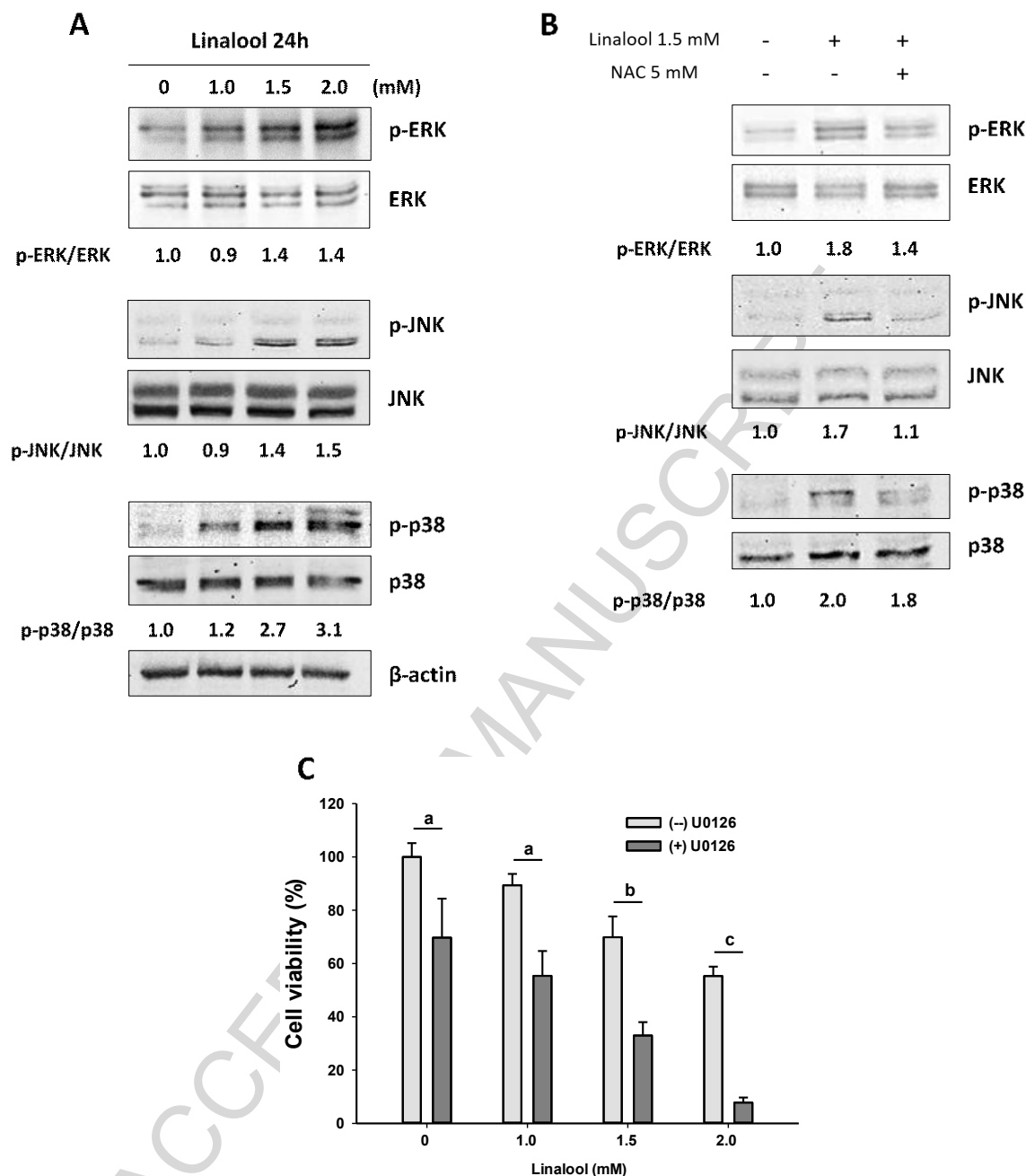


Fig. 7. Effect of linalool on MAPKs phosphorylation. (A) Representative Western blot analysis showing the dose-dependent effects of linalool on ERK, JNK and p38 phosphorylation in HepG2 cells. Each phospho-kinase was normalized to its total kinase. (B) ERK, JNK and p38 expression in HepG2 cells treated with linalool 1.5 mM for 24 h in the absence or presence of NAC 5mM (added 2 h before). Data are expressed as relative change compared to the control group, arbitrarily set at 1 and expressed as the mean of three independent experiments. (C) HepG2 cells were pretreated for

2 h with ERK phosphorylation inhibitor (U0126, 10 μ M) before the addition of linalool (1.0-2.0 mM) for additional 24 h. Cell viability was assessed by WST-1 expression. Data are presented as mean \pm SD (n=8). (a) $p<0.001$; (b) $p<0.00001$; (c) $p<0.000001$.

3.8. Linalool modulates Akt/mTOR pathway

The PI3K/Akt/mTOR represents the major pro-survival anti-apoptotic signaling pathway which promotes chemoresistance of cancer cells against several antineoplastic agents [28]. For this reason, we analyzed the expression of Akt and p70S6K (a surrogate marker for mTOR activation) in response to linalool exposure for 24 h.

Linalool 1.0 and 1.5 mM inhibited Akt phosphorylation by a 55 and 45%, respectively ($p<0.05$), whereas at 2.0 mM, Akt phosphorylation returned to almost control values ($p<0.05$). On the other hand, a significant 80% reduction in p70S6K phosphorylation was observed at 2.0 mM ($p<0.05$) (Fig. 8A,C). Interestingly, a dose-dependent decrease in the total levels of Akt and p70S6K starting from 1.5 mM and 1.0 mM, respectively (Fig. 8B, $p<0.05$), was found. To shed light in Akt phosphorylation recovery at 2.0 mM, HepG2 cells were pre-incubated with Akt phosphorylation inhibitor Ly294002 (Ly, 20 μ M) before addition of linalool for another 24 h. In this context, Ly alone reduced cell viability about 29.5% ($p<0.05$) while in linalool treatment, cell viability was profoundly reduced from 55.3% (linalool alone) to 12.3% (linalool + Ly) (Fig. 8D), suggesting that Akt phosphorylation recovery at linalool 2.0 mM could act as a pro-survival responsive mechanism.

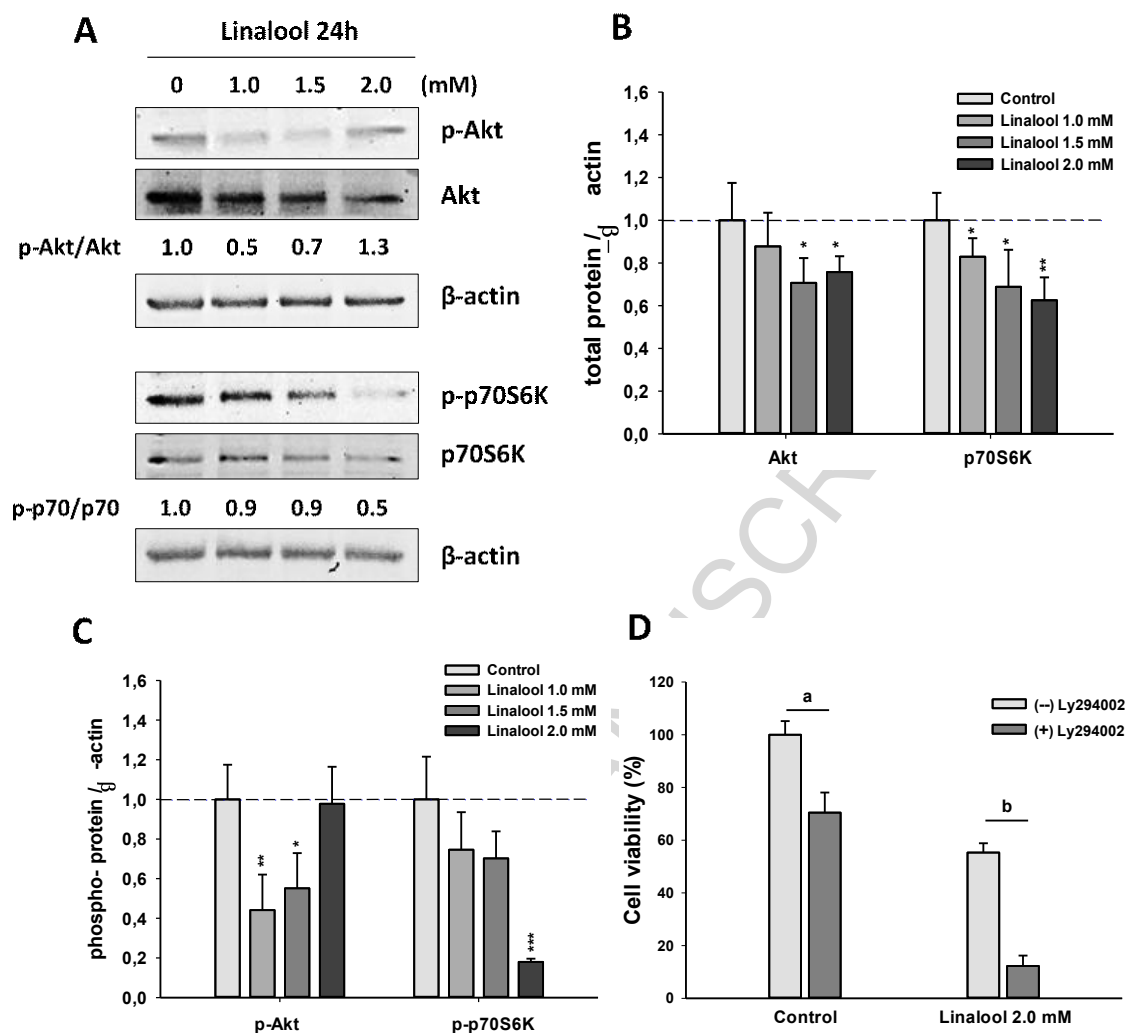


Fig. 8. Effects of linalool on Akt and p70S6 kinases expression. (A) Representative Western blot analysis showing the dose-dependent effects of linalool on Akt and p70S6K in HepG2 cells. Each phospho-kinase was normalized to its total kinase. (B) Immunoblot bands corresponding to total Akt and p70S6K were quantified by densitometric analysis and then normalized to their respective β -actin. Data are expressed as relative change compared to the control group, arbitrarily set at 1 and expressed as the mean \pm SD of three independent experiments. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. (C) Immunoblot bands corresponding to p-Akt and p-p70S6K were quantified by densitometric analysis and then normalized to their respective β -actin. Data are expressed as relative change compared to the control group, arbitrarily set at 1 and expressed as the mean \pm SD of three independent experiments. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. (D) HepG2 cells were pretreated for 2 h with Akt phosphorylation inhibitor (Ly294002, 20 μ M) before the addition of linalool 2.0 mM

for additional 24 h. Cell viability was assessed by WST-1 expression. Data are presented as mean \pm SD (n=8). (a) $p < 0.0001$; (b) $p < 0.000001$.

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4. Discussion

Linalool has been investigated as a promising chemopreventive and chemotherapeutic agent in several *in vitro* and *in vivo* models including liver [25], lung [25], prostate [19], leukemia [20], cervical [21], breast [22], human cancer cells and in tumor-bearing mice [23]. It has been evaluated as a mono-drug agent or combined with conventional drugs, and it was found that antiproliferative concentrations were strongly dependent on cell line in the micro- [19-22] or millimolar [23-25] range. However, the mechanisms by which linalool exerts its antiproliferative effects in HCC cells are not completely understood.

Several monoterpenes including linalool were reported to act as antiproliferative agents against cancer cells by inducing cell growth arrest, both at G0/G1 [21,29] or G2/M [22,30]. In our study, linalool promoted a time and dose-dependent inhibition of cell growth, as indicated by the WST-1 assay. Moreover, particularly at 24 and 48 h, linalool significantly inhibited BrdU incorporation at concentrations lower than those needed for cell viability inhibition, thus demonstrating that linalool was able to specifically inhibit DNA synthesis acting as a cytostatic agent. In addition, we found that linalool promoted a G0/G1 phase cell cycle arrest in HepG2 cells followed by a strong decrease of the cell population in S phase. Although several studies mentioned above focused on linalool activity on cell cycle progression, only a few ones investigated its effect on cell cycle protein modulators [21]. Here we show that linalool modulates the expression of several cell cycle proteins. A marked reduction in Cdk-4 levels was found along with a slight but significant decrease in cyclin E, both of them are crucial for the progression in G1 and transition to S phase [31]. In addition, these changes were accompanied by a strong reduction in cyclin A levels, whose expression peaks in S phase and is dependent on G1/S transition [32], whereas the expression of the p21 and p27 inhibitors, key proteins

inhibiting cell cycle progression to S phase, were increased upon linalool treatments. All these findings are consistent with linalool-induced G0/G1 arrest and DNA synthesis inhibition, ratifying its role as one of the mechanisms by which this monoterpene inhibits HepG2 cell proliferation. The p53 levels, a tumor suppressor protein involved in cell cycle arrest and/or apoptosis in response to cellular stress, significantly decreased after linalool 1.5 and 2.0 mM exposure, therefore suggesting that linalool acts through p53-independent antiproliferative mechanisms. Downregulation of p53 could be attributable to ERK activation, which in HepG2 cells is able to promote p53 degradation via Mdm2-dependent ubiquitination [33].

Linalool was reported to induce apoptosis in several cancer cell lines [19-21,23]. In this study, we show by three different and specific methodologies that linalool induced apoptosis. However, it seemed to contribute to a lesser extent to reduced cell growth than cell cycle arrest, since a moderate caspase-3 activation and an increased proportion of apoptotic cells were only observed at higher concentrations and longer exposure time to linalool.

Ras GTPase is considered a therapeutic target in HCC, given that its downregulation leads to the inhibition of HCC cell growth through cell cycle arrest and/or apoptosis [34,35]. In a previous report [25] we have demonstrated that linalool was able to inhibit HepG2 and A549 cell viability, suggesting that the inhibition of the mevalonate pathway at the level of Ras prenylation, could be one of the antiproliferative mechanisms exerted by linalool, as it was reported for other monoterpenes [6]. Moreover, specific Ras inhibitors containing a 15 carbon isoprene moiety are able to inhibit Ras activity by competing for Ras docking sites at the cell membrane [34]. In the present work, Ras membrane levels were profoundly reduced in response to linalool.

ROS play a complex role in cancer biology. Whereas low levels of ROS normally stimulate cell proliferation, excessive ROS promote irreparable cell damage leading to cell death [36,37]. In fact, ROS appear as one of the most relevant mediators in cell cycle arrest and apoptosis exerted by antineoplastic drugs, including plant-derived natural compounds. Lewinska and collaborators [37] reported that increasing oxidative stress turned from cytostatic autophagy to cytotoxic autophagy leading to apoptosis in breast cancer cells exposed to the flavonoid diosmin. Linalool was shown to promote ROS generation in *in vivo* and *in vitro* cancer models [23]. In the current study, linalool significantly induced a dose-dependent increase in ROS generation from 1.5 mM onwards, which was involved in its antiproliferative activity since pre-incubation with NAC substantially prevented HepG2 cell viability loss. The mechanisms by which linalool promotes ROS generation remain unclear, therefore we analyzed whether NADPH oxidase, a major source of cytoplasmic ROS, was implicated. Apocynin, a specific NADPH oxidase inhibitor, was unable to prevent either ROS generation or cell growth inhibition induced by linalool, suggesting that a mechanism other than NADPH oxidase activity is involved. These results are in line with previous reports proposing that linalool could be able to decrease SOD activity [38] and inhibit mitochondrial complexes I and II [39], subsequently promoting mitochondrial ROS production.

Excessive ROS generation may lead to mitochondria membrane depolarization and, in turn, trigger the apoptotic intrinsic pathway [40]. In our system, linalool induced a mild MMP depolarization after 48 h treatments. The loss of MMP was prevented in the presence of the antioxidant NAC, suggesting that ROS generation promotes and/or exacerbates MMP loss and is probably involved in linalool-induced apoptosis.

Once activated in response to different cellular stimuli, ERK signaling is usually associated with cell survival while JNK and p38 act as tumor suppressors under stress conditions, activating many cellular events like cell cycle arrest, apoptosis and/or autophagy [11,41].

ROS are able to activate the MAPK kinases by different mechanisms, among them through Ras activation, MAPK kinases (MAPKK) activation or MAPK phosphatases inactivation [12,40]. Linalool promoted a strong activation of the three MAPK starting from 1.5 mM, which correlates with the significant increase in ROS generation. Antioxidant NAC totally prevented linalool-induced JNK phosphorylation, pointing to ROS as the source of JNK activation. Moreover, JNK has been identified as a direct promotor of the mitochondrial apoptotic pathway therefore suggesting that ROS-dependent JNK activation is involved in linalool-induced apoptosis, in addition to MMP loss, caspase-3 activation and PARP cleavage. On the other hand, NAC only partially prevented linalool-promoted ERK phosphorylation and had almost no effect on p38 activation. Therefore, it seems that other cellular mechanism than ROS are involved in ERK and p38 induction. In fact, Choi and colleagues [42] reported that ER stress promoted ERK and p38 activation, both involved in autophagy-enhanced cell survival. Indeed, HepG2 cell viability was not completely restored in the presence of NAC suggesting that ROS-independent mechanisms, like p38 induction, Ras downregulation and/or Akt inhibition, could also be involved in linalool-induced cell growth inhibition.

In a context of a cellular stress, ERK activation is often associated with pro-survival functions, however depending on cell type, strength and duration of the signal, its sustained over-activation could promote cell death [12]. In our conditions, ERK activation seems to play a protective role against damage induced by linalool since ERK inhibition mediated by U0126 strongly increased HepG2 cell death. These findings are in line with those reported elsewhere which propose a cytostatic pro-survival autophagy induced by

ERK in response to oxidative injury in cancer cells, which turned into exacerbated cell death after ERK inhibition [36,42]. Moreover, ERK-mediated p53 downregulation presumably could favor HepG2 cells resistance to linalool-induced apoptosis.

It is well known that Akt positively regulates cell cycle progression by several mechanisms, among them, by inhibiting p27 expression and promoting the mTOR/p70S6K axis [43,44]. In the present study, linalool promoted a deep decrease in Akt phosphorylation at 1.0 and 1.5 mM which paralleled p27 upregulation and G0/G1 arrest. On the other hand, the gradual increase in Akt phosphorylation beyond control levels for linalool 2.0 mM occurred in parallel with increased ROS generation and p70S6K inhibition. Akt kinase could be activated in response to excessive ROS production as a consequence of direct Ras activation, PI3K activation and/or a PI3K phosphatase (PTEN) inhibition [40]. mTOR inhibition also triggers Akt activation through a pro-survival negative feedback mechanism [28]. It seems that low concentrations of linalool reduced Akt phosphorylation through lowering Ras and/or PI3K activities, whereas at higher concentrations, linalool induced Akt phosphorylation most likely as a result of ROS generation and/or mTOR inhibition. Indeed, the presence of the specific PI3K/Akt inhibitor Ly294202 co-incubated with linalool 2.0 mM strongly exacerbated cell death, as it was observed in other cell models combining Ly294202 with the specific mTOR inhibitor rapamycin [28]. Altogether, these results illustrate the relevance of the Akt/mTOR pathway in HepG2 cell cycle arrest induced by linalool and support the pro-survival role of Akt phosphorylation recovery. The circumstance that p-p70S6K remained unaltered at linalool 1.0 and 1.5 mM despite Akt inhibition may be due to other kinases activity, such as ERK and/or AMPK, which are able to phosphorylate and modulate mTOR [45]. Interestingly, both Akt and p70S6K protein levels were substantially downregulated after linalool treatment, suggesting that linalool

inhibits Akt/mTOR/p70S6K pathway by mechanisms other than modulating their phosphorylation, which finally contribute to its antiproliferative activity.

Based on the findings of this work, we propose a model for the mechanisms of action of linalool summarized in Fig. 9.

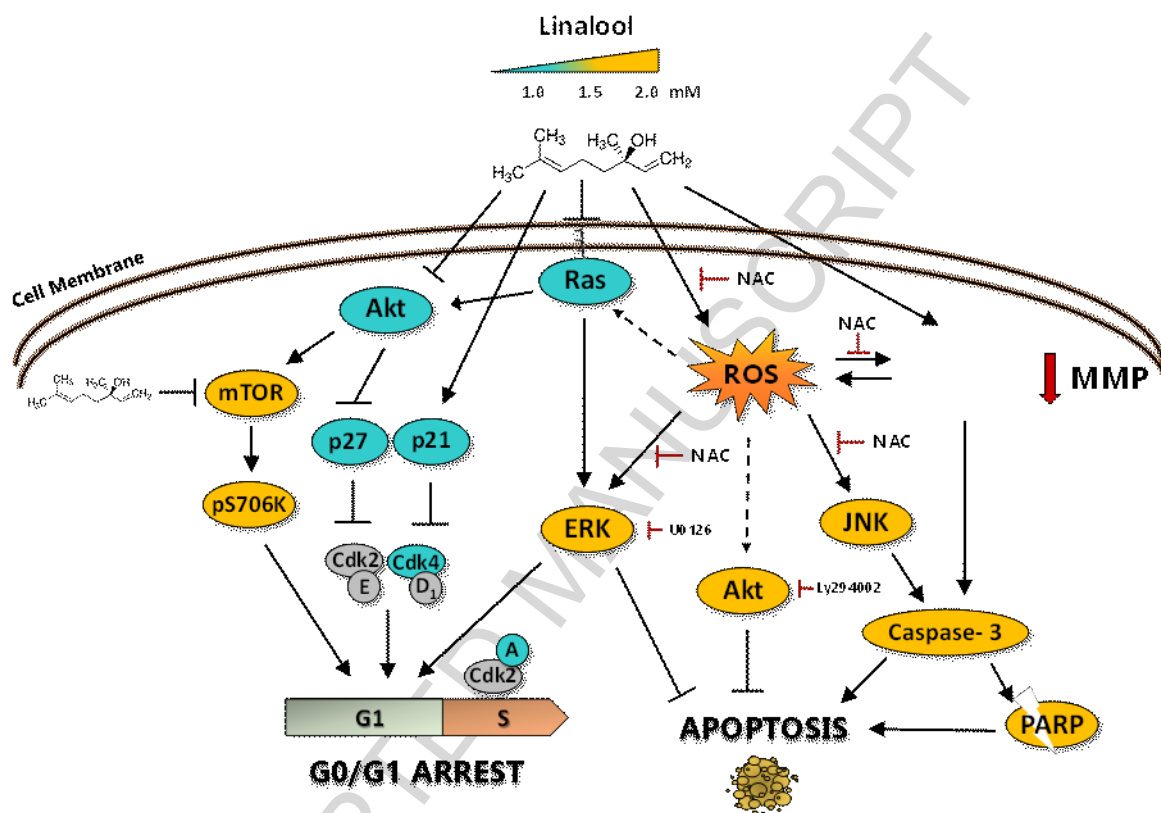


Fig. 9. Proposed model for mechanisms of action involved in linalool-induced HepG2 growth inhibition. Linalool promotes G0/G1 cell cycle arrest from 1.0 mM through Ras and Akt inhibition leading to p27 upregulation, Cdk4 and cyclin A downregulation, as well as by increasing p21 levels (blue proteins). From 1.5 mM it also induces ROS generation which increase at 2.0 mM, promoting Ras, MAPKs activation, MMP depolarization and finally apoptosis through caspase-3 activation and PARP cleavage (orange proteins). In response to cellular stress, ERK and Akt are activated displaying anti-apoptotic/pro-survival roles since their inhibition result in HepG2 cell viability loss. Besides, linalool 2.0 mM also inhibits mTOR/p70S6K which contributes to G0/G1 arrest.

5. Conclusions

Our results demonstrate that linalool inhibits hepatocellular carcinoma HepG2 cells through G0/G1 cell cycle arrest and apoptosis induction. ROS likely play a key role in linalool-induced cytotoxicity in HepG2 cells, which relies on the magnitude and the extent of exposure. For lower concentrations, linalool mainly acts as a cytostatic compound through Akt inhibition, promoting DNA synthesis inhibition by increasing p21 and p27 inhibitors and decreasing Cdk4 and cyclin A proteins. At higher concentrations and a longer exposure time, it also exerts pro-apoptotic activities involving ROS production, MMP depolarization, caspase-3 activation and PARP cleavage. Co-treatment with specific ERK and Akt phosphorylation inhibitors increased linalool-induced HepG2 cell death suggesting protective functions of these kinases in response to cellular stress, thus explaining the observed moderate pro-apoptotic activity of linalool and pointing to both kinases as potential targets for linalool-combination HCC therapies. This work disclosed novel mechanisms involved in the antiproliferative activity of linalool against HepG2 cells and supports the fact that this naturally occurring isoprenoid should be considered as a potential safer chemotherapeutic agent in HCC therapy, employed alone and/or in combination with other chemotherapeutic agents (i.e. ERK inhibitors).

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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Figure Legends

Fig. 1. Linalool inhibited HepG2 cell viability and proliferation. Cells were plated in 96-wells plates and incubated with various concentrations of linalool (0-2.5 mM) for 24, 48 and 72 h. (A) Cell viability assessed by WST-1 expression showing a dose- and time-dependent decrease in HepG2 cell viability. (B) BrdU incorporation into DNA showing a dose-dependent inhibition of HepG2 cell proliferation exerted by linalool. Data are presented as the mean \pm SD (n=8). *p<0.05; **p<0.01; ***p<0.001.

Fig. 2. Linalool induced G0/G1 cell cycle arrest. (A-B) Cell cycle distribution in HepG2 cells after exposed to linalool for 24 h (A) and 48 h (B). Data are expressed as mean \pm SD (n=4). (C) HepG2 cells were incubated with increasing concentrations of linalool for 24 h. Representative immunoblots of cyclin A, cyclin D1, cyclin E, Cdk2, Cdk4, p21, p27, p53 and β -actin (loading control). (D) Quantification of protein levels by densitometric analysis (n=4). Data are expressed as the mean \pm SD. *p<0.05; **p<0.01; ***p<0.001.

Fig. 3. Linalool induced apoptosis in HepG2 cells. HepG2 cells were treated with linalool (1.0, 1.5 and 2.0 mM) for 24 and 48 h and apoptosis was determined by different methodologies. (A) Caspase-3 activity was determined colorimetrically and the results were normalize to control cells (AU= arbitrary units, n=4). Data are presented as the mean \pm SD; *p<0.05; **p<0.01; ***p<0.001. (B) Representative fluorescence micrographs of apoptotic cells labeled by TUNEL assay after 48 h treatments with DMSO 0.1 % (Control) or linalool (LN) at the mentioned concentrations. Cells were examined under an Olympus BX51 fluorescence microscope (magnification \times 10). Upper panel, nuclei stained with DAPI; lower panel, red-stained TUNEL positive cells. (C) Quantification of TUNEL positive cells. About 10-15 fields (100-200 cells/field) of three independent experiments were evaluated. Data are expressed as the mean \pm SD. *p<0.05; **p<0.01; ***p<0.001. (D) Representative immunoblot showing the poly ADP ribose polymerase (PARP) cleavage (apoptotic marker) after 48 h treatment (n=4).

Fig. 4. Effect of linalool on Ras expression. HepG2 cells were treated with linalool (1.0, 1.5 and 2.0 mM) for 24 and 48 h. (A-B) Representative Western Blots of Ras protein in the membrane (mem-Ras) and cytosolic (cyt-Ras) fractions and total Ras after 24 h (A) and 48 h (B) treatments. (C-D) The resulting bands for 24 h (C) and 48 h (D) treatments were quantified by densitometric analysis and then normalized to their respective β -actin. Data are expressed as relative change compared to the control group, arbitrarily set at 1 and expressed as the mean \pm SD of two independent experiments performed by quadruplicate. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

Fig. 5. Oxidative stress induced by linalool. (A-B) HepG2 cells were treated with DMSO 0.1 % (Control), linalool (LN, 1.0, 1.5 and 2.0 mM) or pretreated with NAC 5 mM 2 h prior to addition of linalool 1.5 mM (LN 1.5 mM + NAC) for 3h, and then stained with 10 μ M DCFH-DA for 30 min. (A) ROS production was evaluated by flow cytometry (upper panel). Representative images of HepG2 cells showing ROS positive cells under a fluorescence microscope (20X, lower panel). (B) The fluorescence intensity of the cells analyzed by flow cytometry was calculated relative to that of control (set at 1). Data are expressed as the means \pm SD of three independent experiments (n=4). * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. (C) HepG2 cells were treated with DMSO 0.1 % (Control), LPS (1 μ g/ml) or pretreated with apocynin (Apo, 250 μ M) 2 h prior to addition of LPS (LPS + Apo) or LN 1.5 mM (LN + Apo), and intracellular ROS were detected under a fluorescence microscope (20X). (D) HepG2 cells were pre-incubated with the ROS scavenger NAC (5 mM) or apocynin (250 μ M) 2 h prior to the addition of linalool (1.0 – 2.0 mM) for 24, 48 and 72 h. Cell viability was assessed by WST-1 expression. Data are presented as mean \pm SD (n=8). * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

Fig. 6. Effect of linalool on mitochondrial membrane potential (MMP) in HepG2 cells. (A) HepG2 cells were treated with DMSO 0.1 % (Control) or linalool 1.5 mM at different time points (0-48 h) and MMP was determined by flow cytometry. M1 gates show the percentage MMP loss. Data are expressed as the means \pm SD of two independent experiments (n=4). * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. (B) Representative images of HepG2 cells showing loss of MMP under a fluorescence microscope (40X). Upper panel: cells were treated with DMSO 0.1 % (Control), linalool

1.5 mM at different time points (0-48 h) or pre-incubated with the ROS scavenger NAC (5 mM) 2 h prior to the addition of linalool 1.5 mM. Lower panel: cells were treated for 24 h with DMSO 0.1 % (Control), linalool at different concentrations (1.0 – 2.0 mM) or rotenone 5 μ M (12 h, positive control).

Fig. 7. Effect of linalool on MAPKs phosphorylation. (A) Representative Western blot analysis showing the dose-dependent effects of linalool on ERK, JNK and p38 phosphorylation in HepG2 cells. Each phospho-kinase was normalized to its total kinase. (B) ERK, JNK and p38 expression in HepG2 cells treated with linalool 1.5 mM for 24 h in the absence or presence of NAC 5mM (added 2 h before). Data are expressed as relative change compared to the control group, arbitrarily set at 1 and expressed as the mean of three independent experiments. (C) HepG2 cells were pretreated for 2 h with ERK phosphorylation inhibitor (U0126, 10 μ M) before the addition of linalool (1.0-2.0 mM) for additional 24 h. Cell viability was assessed by WST-1 expression. Data are presented as mean \pm SD (n=8). (a) $p < 0.001$; (b) $p < 0.00001$; (c) $p < 0.000001$.

Fig. 8. Effects of linalool on Akt and p70S6 kinases expression. (A) Representative Western blot analysis showing the dose-dependent effects of linalool on Akt and p70S6K in HepG2 cells. Each phospho-kinase was normalized to its total kinase. (B) Immunoblot bands corresponding to total Akt and p70S6K were quantified by densitometric analysis and then normalized to their respective β -actin. Data are expressed as relative change compared to the control group, arbitrarily set at 1 and expressed as the mean \pm SD of three independent experiments. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. (C) Immunoblot bands corresponding to p-Akt and p-p70S6K were quantified by densitometric analysis and then normalized to their respective β -actin. Data are expressed as relative change compared to the control group, arbitrarily set at 1 and expressed as the mean \pm SD of three independent experiments. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. (D) HepG2 cells were pretreated for 2 h with Akt phosphorylation inhibitor (Ly294002, 20 μ M) before the addition of linalool 2.0 mM for additional 24 h. Cell viability was assessed by WST-1 expression. Data are presented as mean \pm SD (n=8). (a) $p < 0.0001$; (b) $p < 0.000001$.

Fig. 9. Proposed model for mechanisms of action involved in linalool-induced HepG2 growth inhibition. Linalool promotes G0/G1 cell cycle arrest from 1.0 mM through Ras and Akt inhibition (blue proteins) leading to p27 upregulation, Cdk4 and cyclin A downregulation. From 1.5 mM it also induces ROS generation which increase at 2.0 mM, promoting Ras, MAPKs activation, MMP depolarization and finally apoptosis through caspase-3 activation and PARP cleavage (orange proteins). In response to cellular stress, ERK and Akt are activated displaying anti-apoptotic/pro-survival roles since their inhibition result in HepG2 cell viability loss. Besides, linalool 2.0 mM also inhibits mTOR/p70S6K which contributes to G0/G1 arrest.

GRAPHICAL ABSTRACT

