Anti-cancer mechanisms of linalool and 1,8-cineole in non-small cell lung cancer A549 cells

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1. Introduction

Lung cancer is the major cause of cancer-associated deaths worldwide. It has been estimated that lung cancer caused 1.76 million deaths in 2018, accounting for almost one in five (18.4%) of total cancer-related deaths [1]. Lung cancer is classified into two main types: non-small cell lung cancer (NSCLC) and small cell lung cancer (SCLC). NSCLC constitutes about 85% of all lung cancers [2]. Lung cancer presents high mortality/incidence rates (greater than 80%) and poor 5-year survival (10–20%) [3]. Such unfavorable prognosis arises from the highly metastatic behavior of lung cancer cells. Most patients are diagnosed only after they show clinical symptoms in advanced metastases. Moreover, lung cancer cells tend to become resistant to a wide variety of anticancer drugs [4].

Therapeutic strategies for lung cancer include chemotherapy, radiotherapy, surgery, immunotherapy, and targeted therapy. Chemotherapy is the main therapeutic choice, and it is employed both as a first-line option or as adjuvant/neoadjuvant in combined treatments [5, 6, 7, 8]. Conventional chemotherapeutics for NSCLC include etoposide, docetaxel, paclitaxel, cisplatin, irinotecan, and doxorubicin among others, which are generally administered in pairwise combinations [8]. The use of drug combinations is intended to enhance the individual effect of each one as well as to minimize or avoid potential tumor resistance to monotherapy, which is recurrent in NSCLC [9]. Also, conventional chemotherapy presents severe toxic side effects such as hair loss, nausea and vomiting, diarrhea, loss of appetite, leukocyte decrease, and tiredness, leading to considerable disruption of the treatments [10, 11]. These undesirable effects are caused by the poor selectivity of the antineoplastic
drugs for malignant tissue over normal tissue. Consequently, antineo-
plastic agents inhibit not only the proliferation of cancer cells but also of
normal cells with a high rate of division -hematopoietic precursors, epithelial cells, and hair cells. [10]

Looking for alternatives to replace or complement the current che-
motherapeutics, phytochemicals arise as natural, abundant, low-cost,
and low- or even non-toxic molecules with various pharmacological
properties including anticancer activity [12, 13, 14]. Monoterpenes are
aromatic 10-carbon isoprenoids isolated from essential oils present in
plants. These molecules are widely used in the food industry and cos-
metics and are recognized as safe (GRAS) by the Food and Drug
Administration (FDA, USA). Many studies have shown the antitumor
activity of monoterpenes in cancer cells in vitro and in vivo through
promoting cell cycle arrest, apoptosis, autophagy, and/or senescence
[15, 16, 17, 18]. The main anticancer mechanisms reported for these
natural compounds include the inhibition of proteins involved in cell
survival and proliferation (Ras, ERK, Akt), modulation of oxidative stress
and endoplasmic reticulum stress, and depolarization of mitochondrial
membrane potential [18, 19, 20]. In addition, it was reported increased
anticancer activity of antineoplastic drugs combined with monoterpenes,
suggesting their potential as novel anticancer molecules also employed as
sensitizer compounds [21, 22, 23].

Linalool is a linear monoterpene present in over 200 species of plants
like coriander, basil, laurel, cinnamon, grapevine, green and black teas
[24]. 1,8-cineole (eucalyptol), is a cyclic-ether monoterpene found in
several plants such as eucalyptus, rosemary, sage, bay, cinnamon, and tea
[24]. Both monoterpenes showed pharmacological properties including
analgesic, sedative, antioxidant, anti-inflammatory, bactericidal, fungal-
cidal, hypolipidemic, and anticancer activities [25, 26, 27]. We have
previously shown for the first time that linalool and 1,8-cineole inhibit
the proliferation of NSCLC A549 cells [28]. Moreover, we demonstrated
that the combination of each monoterpene with simvastatin - a
lipid-lowering drug with high potential in drug repurposing for cancer
treatment-synergistically inhibited A549 cell proliferation [28]. How-
ever, the mechanisms involved remained elusive.

The present study aimed to explore the anticancer mechanisms of
linalool and 1,8-cineole, alone or each one in combination with simva-
statin, on lung adenocarcinoma A549 cells.

2. Materials and methods

2.1. Reagents and antibodies

(-)-linalool (>95%, Figure 1A), 1,8-cineole (>99%, Figure 1B), sim-
vastatin, 2,7-dichlorodihydrofluorescein diacetate (DCFH-DA), N-acetyl-
L-cysteine (NAC), rhodamine-123, and dimethyl sulfoxide (DMSO) were
purchased from Sigma Chemical Co. (St Louis, MO, USA). Dulbecco’s
Minimal Essential Medium (DMEM), and penicillin/streptomycin were
obtained from Gibco (Invitrogen Corporation, USA). Protease inhibitors
were from Thermo Scientific (Rockford, USA). Antibodies for PARP,
caspase-3, and caspase-9 were from Santa Cruz Biotechnology (Santa
Cruz, CA, USA). Anti-GAPDH antibody was from Sigma Aldrich Co (St
Louis, MO, USA).

2.2. Cell culture

Human lung adenocarcinoma A549 cells were obtained from the
American Type Culture Collection (ATCC CCL-185). WI-38 cells (normal
human embryonic lung fibroblasts, ATCC CCL-75) were a kind gift from
Dr. Natalia Scaglia (School of Medicine, National University of La Plata).
Both cell lines were maintained in Dulbecco’s modified Eagle’s medium
(DMEM) supplemented with 10% (v/v) fetal bovine serum (Natocor,
Argentina) and 1% penicillin/streptomycin (5% CO2 at 37 °C). The
medium was replaced every 2–3 days until cells were passaged after
reaching 80–90% confluence. In experiments, cells were cultured under
standard conditions for 24 h before the beginning of the treatments.
Then, the cells were exposed to DMEM supplemented with 0.2% ethanol
(vehicle), 1,8-cineole, or linalool for up to 48 h. The medium was
replaced every 24 h. Linalool and 1,8-cineole were dissolved in ethanol

![Figure 1](https://example.com/figure1.png)

**Figure 1.** Effect of linalool (A) and 1,8-cineole (B) on A549 and WI-38 cell viability. Cells were exposed to increasing concentrations of linalool (C) or 1,8-cineole (D) for 24 and 48 h. Cell viability was determined by the MTT assay. Data are expressed as means ± SD. *p < 0.05; **p < 0.01; ***p < 0.001.
0.2%, a concentration of vehicle that does not affect cellular viability or metabolic activity of A549 cells.

2.3. Cell viability

A549 (4 × 10³) and WI-38 (8 × 10³) cells seeded in 96-well plates were treated with DMEM supplemented with ethanol 0.2%, linalool (0.03 mM) or 1,8-cineole (0–10 mM) for 24 or 48 h. To evaluate the role of oxidative stress on cell viability, A549 cells were pre-incubated or not with the antioxidant NAC (5 mM) for 2 h prior to the addition of linalool (1.0 and 2.0 mM), 1,8-cineole (4.0 and 8.0 mM), or hydrogen peroxide 0.25 mM (positive control) for 24 h.

Cell viability was determined by the MTT assay (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) [29]. After treatments, the cells were washed twice in PBS and incubated with MTT solution (0.5 mg/ml in PBS) for 3 h. Formazan was dissolved in 0.1 ml DMSO, the plates were shaken for 10 min, and the absorbance at 560 nm measured with a microplate reader (Beckman Coulter DTX 880).

2.4. Cell cycle analysis

Cells were plated in 6-well plates at a density of 1.5 × 10⁵ and allowed to adhere 24 h. Then, the cells were exposed to ethanol 0.2%, linalool (1.0 and 2.0 mM), 1,8-cineole (4.0 and 8.0 mM), simvastatin 10 μM, or the combination of each monoterpene (linalool 1.0 mM or 1,8-cineole 4.0 mM) with simvastatin 10 μM for 24 h. After treatments, the cells were harvested, centrifuged at 500 × g for 5 min, resuspended in PBS (NaCl 137 mM; KCl 2.7 mM, 10.0 mM Na₂HPO₄, 2.0 mM KH₂PO₄, pH 7.4), and fixed in 70% (v/v) ice-cold ethanol at 4 °C overnight. After washing twice with cold PBS, the cells were incubated with RNase A (500 U/ml; Biodynamics, Argentina) at 37 °C for 20 min. Nuclei were stained with propidium iodide (0.025 mg/ml PI in PBS containing 0.1% (v/v) Triton X-100) in the dark for 30 min. The stained cells were detected with a FACSAria II flow cytometry cell sorter (Becton Dickinson, California, USA) and the cell cycle distribution was analyzed by FlowJo 7.6.2 (Tree Star, Oregon, USA).

2.5. Cell death

A549 (4 × 10³) cells were seeded in 96-well plates and treated with DMEM supplemented with ethanol 0.2%, linalool (1.0 and 2.0 mM), or 1,8-cineole (4.0 and 8.0 mM) for 24 or 48 h. Cell death was determined by trypan blue assay as previously described [28]. Live (trypan blue-excluding) and death (blue-stained) cells were counted in a Neubauer hemocytometer.

2.6. In situ detection of apoptosis

A549 cells (1.5 × 10⁵) plated on sterile coverslips (24 × 24 mm) in 6-well plates were incubated for 48 h with ethanol 0.2%, DMSO 5% (positive control), linalool (1.0 and 2.0 mM), 1,8-cineole (4.0 and 8.0 mM), simvastatin (10, 25 and 50 μM), or the combination of each monoterpene (linalool 1.0 mM or 1,8-cineole 4.0 mM) with simvastatin 10 μM. DMSO 5% was chosen as a positive control for apoptosis in A549 cells based on the literature available [30]. Apoptosis was determined by dUTP deoxynucleotidyl transferase-mediated nick end labeling (TUNEL) assay (In-Situ Cell Death Detection Kit, TMR Red, TRC Red, Mannheim, Germany) following the manufacturer’s protocol. Then, the samples were mounted with ProLong® Gold Antifade Reagent containing 4',6-diamidino-2-phenylindole dihydrochloride (DAPI, Invitrogen, Life Technologies, Carlsbad, CA, USA). The TUNEL-positive cells were observed under an Olympus BX51 Fluorescence Microscope (Olympus, Tokyo, Japan) and images were examined by means of ImagePro Plus v. 5.1 software (Media Cybernetics, Silver Spring, MD).

2.7. Determination of reactive oxygen species (ROS)

Intracellular ROS were determined using DCFH-DA (Sigma Aldrich) as previously described [16]. A549 cells seeded in 24-well plates (2.5 × 10⁴) were exposed for 24 h to ethanol 0.2%, linalool (1.0 and 2.0 mM), 1,8-cineole (4.0 and 8.0 mM), hydrogen peroxide 0.25 mM (positive control), or pre-incubated with 5 mM NAC for 2 h before the addition of linalool 2.0 mM or 1,8-cineole 8.0 mM. Following, the cells were stained with 10 μM DCFH-DA in serum-free DMEM in the dark at 37 °C for 30 min. After washing three times with PBS, the cells were analyzed under an Olympus LX71 Inverted Fluorescence Microscope (Olympus, Tokyo, Japan) at an excitation wavelength of 488 nm and an emission wavelength of 525 nm.

2.8. Evaluation of mitochondrial membrane potential (MMP)

MMP was measured using the fluorescent dye rhodamine-123, as previously described [16]. A549 cells were seeded in 24-well plates (2.5 × 10³) and treated for 24 h with linalool (1.0 and 2.0 mM), 1,8-cineole (4.0 and 8.0 mM), hydrogen peroxide 0.25 mM, rotenone 2 μM (positive control), or pre-treated with NAC (5 mM, 2 h) before being exposed to linalool 2.0 mM or 1,8-cineole 8.0 mM. Then, the cells were washed three times with PBS, stained with 5 μM rhodamine-123 in free-serum DMEM for 30 min at 37 °C in the dark. After washing three times with PBS, cells were imaged under an Olympus LX71 Inverted Fluorescence Microscope (Tokyo, Japan) at 488/525 nm (ex/em). The fluorescence intensity was measured using ImageJ software 1.52 a.

2.9. Wound healing assay

A549 cells were seeded in 24-well plates at a density of 7.5 × 10⁴ for 24 h. The monolayers were scratched with a sterile yellow pipette tip and washed with serum-free DMEM to remove the detached cells. Then, the cells were treated with ethanol 0.2%, linalool (1.0 and 2.0 mM) or 1,8-cineole (4.0 and 8.0 mM) dissolved in serum-free DMEM. The migration of A549 cells was observed under an Olympus LX71 Inverted Fluorescence Microscope (Tokyo, Japan) at 0 and 48 h after wounding. The wound healing area was measured by Image J 1.51k software (National Institute of Health, USA).

2.10. Western blot analysis

A549 cells (6 × 10⁵) were seeded in 100 mm Petri dishes and allowed to attach 24 h before being treated with ethanol 0.2% (control), linalool 2.0 mM, 1,8-cineole 8.0 mM, or DMSO 5% for 48 h. Then, the cells were rinsed twice with PBS and lysed in RIPA lysis 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)] supplemented with protease inhibitors (Thermo Fisher Scientific, USA; Cat. #A32963) for 30 min on ice and centrifuged 15 min at 14,000 × g at 4 °C. The protein content was quantified by the method of Bradford [31]. Protein samples (50 μg) were subjected to Western Blot according to standard procedures previously reported [16]. GAPDH was employed as a loading control.

2.11. Statistics

Results are expressed as a relative change compared with ethanol 0.2% controls and presented as means ± SD. Statistical analysis was performed through the use of the one-way analysis of variance (ANOVA) and the Tukey–Kramer multiple-comparisons test or the unpaired t-test with the significance level set at p < 0.05 (GraphPad inStat program). The IC50 values for cell viability were calculated by nonlinear-regression curves (SigmaPlot software; Systat Software, Inc., Point Richmond, CA).
3. Results

3.1. Effect of linalool and 1,8-cineole on A549 and WI-38 cell viability

To investigate the effects of linalool and 1,8-cineole on cancer A549 and normal WI-38 lung cell growth inhibition, the MTT assay was performed. Both monoterpenes promoted a dose- and time-dependent inhibition on A549 cells. Linalool inhibited cell growth starting from 1.0 mM at 24 and 48 h (Figure 1C, p < 0.05) whereas 1,8-cineole significantly decreased cell viability from 8.0 mM after 24 h, and from 4.0 mM after 48 h, respectively (Figure 1D, p < 0.05 in both cases). On the other hand, linalool concentrations up to 2.0 mM incubated for 24 and 48 h were not cytotoxic for WI-38 cells (Figure 1C) meanwhile 1,8-cineole slightly decreased WI-38 cell viability only from 8.0 mM after 48 h incubation (Figure 1D). The IC50 values obtained are summarized in Table 1. Based on these results, linalool 1.0 and 2.0 mM and, 1,8-cineole 4.0 and 8.0 mM were selected for the following experiments since they inhibit the growth of A549 tumor cells without significant effects on normal WI-38 cells.

3.2. Linalool and 1,8-cineole induced A549 cell cycle arrest

To gain some insight into the antiproliferative mechanisms of linalool and 1,8-cineole, cell cycle analysis was carried out by flow cytometry (Figure 2A). Both monoterpenes induced a strong G0/G1 arrest. Linalool increased G0/G1 population from 58.1% in control cells to 82.7 and 81.4% after incubated with linalool 1.0 and 2.0 mM, respectively (Figure 2B, p < 0.001). This effect was accompanied by a reduction in S-phase cells from 37.8% to 11.6 and 14.6%, respectively (p < 0.001). No significant changes were found in the G2/M phase and Sub-G1 cells. A similar profile was obtained in 1,8-cineole-treated cells, but in this case, the number of G2/M cells significantly augmented after 8.0 mM incubation (4.2% in control cells to 13.5% in treated cells, p < 0.05) (Figure 2B). These results show that linalool and 1,8-cineole present cytostatic effects in A549 cells and no apoptotic (Sub-G1) cells are detected after 24-h exposure.

3.3. Linalool and 1,8-cineole did not trigger apoptosis in A549 cells

Given that none of each monoterpene increased Sub-G1 cell population (Figure 2B) or induced the activation of caspase-3 (data not shown) after 24 h, we next evaluated the capability of linalool and 1,8-cineole to induce apoptosis in A549 cells after 48 h treatments. First, we analyzed DNA fragmentation—a hallmark of late apoptosis by TUNEL assay. As shown in Figure 3A-B, none of both monoterpenes was able to trigger apoptosis, even in conditions where cell viability was inhibited up to 80–85% (linalool 2.0 mM or 1,8-cineole 8.0 mM, 48 h). Then, to confirm these observations, we analyzed the cleavage (activation) of caspase-3 (an effector caspase induced by both intrinsic and extrinsic pathways), caspase-9 (the initiator caspase of the intrinsic pathway), and PARP (a substrate for effector caspases) by western blot. As observed in Figure 3, no cleaved caspase-3 (Figure 3C) or changes in procaspase-9 and PARP levels (Figure 3D) were found. These results show that none of both monoterpenes induced apoptosis.

To determine whether linalool or 1,8-cineole induce cell death in A549 cells, the trypan blue exclusion assay was performed (Figure 3E). Linalool 2.0 mM increased cell death from about 2.8 and 3.0% in control cells to 9.6 and 12.9% after 24 and 48 h, respectively (p < 0.001). On the other hand, 1,8-cineole did not induce cell death in any experimental condition. The increase in trypan blue-stained cells but not in TUNEL-positive cells suggests that linalool may induce cell death types distinct from apoptosis in A549 cells.

3.4. Effects of linalool and 1,8-cineole on oxidative stress and ROS-induced cell growth inhibition

We then studied whether linalool and 1,8-cineole trigger ROS generation. Figure 4A shows that linalool, but not 1,8-cineole, was able to induce ROS production in A549 cells, even from low concentrations such as 1.0 mM linalool. Moreover, pre-treatment with the antioxidant NAC prevented such an increase in 2.0 mM linalool-treated cells and improved the number of A549 cells in comparison to non-pre-treated cells (Figure 4A). To gain understanding of whether ROS production was involved in the antiproliferative activity, A549 cells were exposed to linalool or 1,8-cineole in the absence or presence of NAC for 24 h and cell viability was assessed. As shown in Figure 4B, NAC prevented cell viability loss from 65.9 and 36.4% to 85.2 and 59.2% in linalool 1.0- and 2.0-mM treated cells, respectively (p < 0.001). As expected, NAC failed to prevent cell growth inhibition induced by 1,8-cineole (Figure 4B).

These results evidence that ROS partially contribute to the antiproliferative effects of linalool but are not involved in 1,8-cineole-induced cell growth inhibition.

3.5. Linalool, but not 1,8-cineole, induced depolarization of mitochondrial membrane potential (MMP)

Mitochondria are the primary source of ROS in the cell and, at the same time, excessive ROS promote mitochondrial damage and MMP collapse. Both oxidative stress and mitochondrial dysfunction may contribute to antiproliferative processes other than apoptosis. Consequently, MMP was evaluated through the incorporation of rhodamine-123 (Figure 5A). We found that linalool 1.0 and 2.0 mM induced a significant decrease of 17.7 and 47.6% in MMP, respectively (Figure 5B). NAC prevented to some extent the MMP collapse promoted by linalool 2.0 mM (Figure 5B, p < 0.001), as observed by greater green fluorescence intensity of A549 cells when pre-incubated with the antioxidant molecule (Figure 5A). On the other hand, 1,8-cineole did not cause any significant effect on MMP (Figure 5 A-B).

These findings show that linalool induced the depolarization of MMP in A549 cells, which may be involved in cell viability loss, and suggest that linalool-induced oxidative stress is responsible for MMP depolarization.

3.6. Linalool and 1,8-cineole inhibited A549 cell migration

To expand the knowledge on the anti-cancer potential of linalool and 1,8-cineole in A549 cells, the effect on cell migration was studied through the wound healing assay. We found that after 48 h, both monoterpenes significantly suppressed A549 cell migration at the higher concentrations employed (Figure 6A). Linalool 2.0 mM and 1,8-cineole 8.0 mM reduced the wound healing closure to 61.7 and 62.5%, respectively, in comparison to the 77.9% observed in control cells (Figure 6B, p < 0.01 in both cases). These results suggest that both isoprenoids also exert anti-metastatic effects on NSCLC A549 cells.

Table 1. IC50 values of linalool and 1,8-cineole on A549 and WI-38 cells.

|         | Linalool | 1,8-cineole |
|---------|----------|-------------|
| A549    | 24 h     | 48 h        |
|         | 1.79 ± 0.11 | 1.13 ± 0.09 |
|         | 8.30 ± 0.26 | 5.84 ± 0.21 |
| WI-38   | >3       | >3          |
|         | >10      | >10         |
Figure 2. Linalool and 1,8-cineole induced A549 cell cycle arrest. A549 cells were exposed to ethanol 0.2% (Control), linalool 1.0 (LN 1) and 2.0 (LN 2) mM, or 1,8-cineole 4.0 (CN 4) and 8.0 (CN 8) mM for 24 h. The cell cycle was analyzed by flow cytometry (A) and quantified (B). Data are expressed as mean ± SD (n = 4). ***p < 0.001.
3.7. Linalool and 1,8-cineole sensitized A549 cells to simvastatin-induced cell cycle arrest and apoptosis

In a previous study, we demonstrated that pairwise combinations of linalool or 1,8-cineole with simvastatin synergistically inhibited A549 cell growth [28]. Here, we evaluated the mechanisms by which linalool and 1,8-cineole act as sensitizer molecules when combined with simvastatin. We analyzed the effect of the pairwise-combination of simvastatin and each monoterpene (linalool 1.0 mM or 1,8-cineole 4.0 mM) on cell cycle progression and apoptosis (Figure 7). We used simvastatin 10 μM, a sub-IC50 concentration that inhibited A549 cell viability by 16.4 and 27.8% after 24 and 48 h, respectively (Figure A1). This concentration had moderate effects on the cell cycle but did not induce apoptosis in A549 cells (Figure 7).

As shown in Figure 7A, the combined treatments increased the G0/G1 cell population and decreased S-phase cells compared to individual treatments, resulting in both cases, statistically different from cells treated with simvastatin alone (p < 0.05). Thereafter, we investigated the effects of the combination of each monoterpene and simvastatin on apoptosis (Figure 7B). As observed for simvastatin 10 μM (Figure 7C), and linalool 1.0 mM and 1,8-cineole 4.0 mM (Figures 3 and 7C), none of the three compounds employed alone showed to induce apoptosis.
Figure 4. Effects of linalool and 1,8-cineole on intracellular reactive oxygen species (ROS) in A549 cells. (A) Cells were incubated with ethanol 0.2% (Control), linalool 1.0 (LN 1) and 2.0 (LN 2) mM, 1,8-cineole 4.0 (CN 4) and 8.0 (CN 8) mM, hydrogen peroxide 0.25 mM (H₂O₂, positive control), or pre-incubated with NAC 5 mM 2 h prior to treatment with linalool 2.0 mM (LN 2 + NAC) or 1,8-cineole 8.0 mM (CN 8 + NAC) for 24 h. ROS production was analyzed under an Inverted Fluorescence Microscope. Scale bar, 50 μm. (B) Effect of the antioxidant NAC on A549 cell viability. Cells were pre-incubated or not for 2 h with NAC 5 mM before the addition of ethanol 0.2%, LN 1, LN 2, CN 4, CN 8, or H₂O₂ for 24 h. Cell viability was determined by the MTT assay. Data are presented as the mean ± SD (n = 12). ***p < 0.001.
Figure 5. Effects of linalool and 1,8-cineole on mitochondrial membrane potential (MMP) in A549 cells. (A) Cells were incubated with ethanol 0.2% (Control), linalool 1.0 (LN 1) and 2.0 (LN 2) mM, 1,8-cineole 4.0 (CN 4) and 8.0 (CN 8) mM, hydrogen peroxide 0.25 mM (H2O2), or rotenone 2 μM (ROT, positive control) for 24 h and then stained with rhodamine-123. (A) Cells were observed and photographed under a fluorescence microscope. Scale bar, 50 μm. (B) Quantitative analysis of rhodamine-123 green fluorescence in A549 cells expressed as a percentage of MMP loss compared to control cells. Data are shown as mean ± SD (n = 6). *p < 0.05; **p < 0.01; ***p < 0.001 vs. Control; #p < 0.001 vs. LN 2.
compared to control cells. However, the combination of each monoterpene and simvastatin showed a significant 3- to 5-fold increase in TUNEL-positive cells respect to control cells and individual treatments (Figure 7C, \( p < 0.05 \)). These results were confirmed by the detection of cleaved PARP in the combined treatments (Figure 7D).

4. Discussion

Nature is an excellent source of molecules with antitumor activity. Approximately 60% of the anticancer drugs currently available for clinical use come from natural sources, including most of the antineoplastic drugs against NSCLC [32]. Linalool and 1,8-cineole have been evaluated as potential chemopreventive and chemotherapeutic molecules as mono-drug agents or combined with conventional drugs. Numerous \textit{in vitro} and \textit{in vivo} studies demonstrated the anticancer potential of these monoterpenes in lung [28], liver [16, 26, 33], breast [23], ovarian [34], skin [35], cervical [56, 37], colon [38, 39], sarcoma [40], glioma [41], leukemia [26, 42, 43], prostate [44, 45], oral [46] and gastric [47] cancer cells. Nevertheless, to our knowledge, the mechanisms by which linalool and 1,8-cineole impair the proliferation of NSCLC A549 cells remain unexplored. One of the main and most difficult goals in cancer drug discovery is to obtain molecules with preferential activity toward cancer cells, with no or minimal toxicity against normal tissues. In this study, we show that linalool (1.0–2.0 mM) and 1,8-cineole (4.0–8.0 mM) significantly inhibited A549 cell viability without showing inhibitory effects on normal lung WI-38 cells. The effective concentrations of linalool and 1,8-cineole reported to inhibit \textit{in vitro} cancer cell proliferation vary from micromolar (\( \mu M \)) [35, 36, 39, 42, 43, 45, 47] to millimolar (mM) [16, 33, 34, 37, 38, 40, 44, 46]. This heterogeneity in sensitivity depends on the cancer cell type but also each cell line evaluated. For example, leukemia cells are between the most sensitive cells to both monoterpenes [36, 42, 43]. Interestingly, Gu and collaborators [43] found that the activity of linalool strongly depended on the p53 status of a panel of leukemia cells, obtaining IC50 ranging from 80 \( \mu M \) (p53 wild-type) to greater than 640 \( \mu M \) (mutated p53). Several studies employing linalool [34, 40, 44], 1,8-cineole [38], or other monoterpenes [15, 48] at millimolar concentrations \textit{in vitro}, showed strong antitumor activity \textit{in vivo} at non-toxic doses (10–400 mg/Kg). Among them, Jana et al. [40] required \( \sim 2.0 \) mM linalool to inhibit 50% cell viability in S-180 sarcoma cells \textit{in vitro}. When they orally administered 200 mg/kg linalool in mice bearing S-180 tumor, a concentration of 102 ng/ml (0.66 \( \mu M \)) linalool was measured in sera after 18 h, which is much lower than used in \textit{in vitro} cultured cells. However, such treatment applied every 48 h for 3 weeks drastically reduced tumor growth by 50%.
Cell cycle arrest is considered one of the major causes of cell growth inhibition. Numerous monoterpenes, among them linalool and 1,8-cineole, showed cytostatic antiproliferative effects against cancer cells by causing G0/G1 [33,36] or G2/M [35, 49] cell growth arrest. In this study, 1,8-cineole appeared to affect cell cycle distribution in a different manner depending on the concentration. 1,8-cineole induced a robust G0/G1 arrest along with a considerable decrease of S-phase cells at both concentrations. However, at 8.0 mM, it also raised the proportion of cells in the G2/M phase and partially diminished G0/G1 cells respect to the lower concentration (4.0 mM). On the other hand, linalool induced a marked G0/G1 cell cycle arrest from 1.0 mM onwards, accompanied by a strong diminution of S-phase cells, as observed previously in liver cancer cells [33].

Apoptosis is one of the chief strategies for cancer therapy exerted by most chemotherapeutic agents. It has been reported the ability of linalool and 1,8-cineole to induce apoptosis in a wide variety of cell lines, including skin [35], oral [46], sarcoma [40], and liver [33] among others. In this study, several apoptotic markers such as sub-G1 cells, DNA fragmentation, and cleavage of caspase-3, caspase-9, or PARP were evaluated. Even exposing cells to concentrations higher than IC50, neither linalool nor 1,8-cineole induced apoptosis in A549 cells. Many natural compounds with anticancer effects showed similar results in this

Figure 7. Linalool and 1,8-cineole increased simvastatin-mediated cell cycle arrest and apoptosis in A549 cells. Cells were exposed to ethanol 0.2% (Control), linalool 1.0 mM (LN 1), 1,8-cineole 4.0 mM (CN 4) and simvastatin 10 μM (SV) alone or each monoterpane combined with simvastatin (LN + SV, CN + SV). (A) The cell cycle was analyzed following 24 h treatments. Scale bar, 10 μm. (B) Representative images of TUNEL assay performed after exposing A549 cells to the treatments for 48 h. (C) Around a total of ten to fifteen fields (200-500 cells/field) were evaluated and TUNEL-positive cells were quantified. Data are expressed as mean ± SD. Means without the same letter are different (p < 0.05). (D) Representative western blot of PARP and cleaved-PARP. GAPDH was used as a loading control.
NSCLC cell line, suggesting that mechanisms of cell death other than apoptosis (autophagic cell death, necroptosis, ferroptosis, etc.) mediate their antitumor action [50, 51]. Here, we observed that linalool, but not 1,8-cineole, induced modest cell death. 1,8-cineole likely mediates only cytostatic activity in A549 cells, which could be indicative of developing senescence, a permanent cell cycle arrest [52]. Some compounds induce autophagy before the establishment of the senescent phenotype, a process that may require several days [16, 52]. Indeed, we have previously shown that 1,8-cineole induced AMPK activity, an energy sensor protein that promotes autophagy [17], before developing senescence in hepatocarcinoma HepG2 cells [16]. 1,8-cineole induced AMPK in A549 cells, but no senescence markers like p21-or nuclear size increase were observed in our conditions (data not shown). Nevertheless, additional features and longer-time experiments are needed to confirm whether autophagy and/or senescence are involved.

ROS play a key role in multiple signaling pathways that are crucial to the fate of both healthy and cancer cells [53]. Mitochondria are the major endogenous source of ROS in human tissues and produce them at physiological levels as a natural byproduct of oxidative phosphorylation. Cancer cells show increased levels of ROS than their normal counterparts, mainly as a consequence of the augmented metabolic activity in mitochondria, alterations in the electronic transport chain, and oncogenic signaling in cancer cells [53, 54]. The high levels of ROS in cancer cells are associated with tumorigenesis, angiogenesis, metastasis, and tumor resistance [53, 54]. Increasing ROS levels to highly toxic amounts activates several ROS-dependent cell death pathways. A common strategy followed by several anticancer agents -including phytochemicals-is targeting mitochondria, leading to ROS generation and mitochondrial dysfunction [55]. Elevation of ROS in moderate quantities may induce autophagic cell death, cell cycle arrest, and senescence in cancer cells, whereas greater amounts of ROS promote cytotoxic cell death [52]. Moreover, oxidative stress and mitochondrial collapse may contribute to cell death independent from apoptosis through autophagic cell death, necroptosis, or ferroptosis, among others [52, 56, 57]. Linalool, 1,8-cineole, and a wide variety of other monoterpenes exerted their anticancer action through ROS production and MMP depolarization [16, 33, 34, 35, 39, 40, 47]. Here, we found that linalool, but not 1,8-cineole, induced ROS production in A549 cells. Furthermore, the presence of the antioxidant NAC reverted to some degree the linalool-induced cell growth inhibition in A549 cells but had no effect on the antiproliferative action of 1,8-cineole. Besides, only linalool promoted a significant loss in MMP and the preincubation with NAC rescued the cells from such depolarization. Linalool likely has some effect on mitochondrial components promoting the generation of mitochondrial ROS, which in turn favor the mitochondrial collapse, as previously described elsewhere [33, 41, 58].

NSCLC A549 cells have shown a marked resistance to apoptosis induction mediated by several anticancer agents. Such resistance was associated with the high expression of proteins of the antioxidant system in this cell line [59]. This fact may play a role in the inability of 1,8-cineole to induce oxidative stress and of both monoterpenes to trigger apoptosis in A549 cells, unlike what was observed in other cell lines [33, 35, 38].

Cell migration is a key feature of cancer metastasis. This is a complex biological process that allows cancer cells to invade the neighboring tissues and spread to other body parts, thus promoting metastases [60]. The anti-migratory effects of phytochemicals have been widely explored in highly metastatic cells, including NSCLC cells [18, 61]. Nonetheless, to our knowledge, no studies explored the ability of linalool and 1,8-cineole to inhibit cancer cell migration. Here, we observed that both compounds reduced A549 cell migration at the higher concentrations tested.

The need for new and more effective treatments against cancer encouraged researchers to study new uses for affordable drugs approved for other diseases, a strategy known as “drug repurposing”. Statins are hypolipidemic drugs with high potential as anticancer agents, particularly in cancer cells with high 3-hydroxy-3-methylglutaryl coenzyme A reductase -the target enzyme of statins- and Ras activity, as is the case of NSCLC A549 cells [62, 63]. Monoterpenes showed to potentiate the anticancer activity of statins and to reduce the statin-associated adverse effects, thus proposed as adjuvant molecules in statin-based chemotherapies [63]. We have previously shown the antiproliferative synergistic effects of the combination of linalool or 1,8-cineole with simvastatin [28]. Here, we unraveled the effects of these combinations on cell cycle progression and apoptosis in A549 cells. Linalool and 1,8-cineole significantly increased cells arrested in G0/G1 cells when combined with simvastatin respect to A549 cells exposed to the statin alone. Moreover, both monoterpenes sensitized cells to simvastatin-induced apoptosis. The sum of the effects on cell cycle and apoptosis may account for the antiproliferative synergism previously reported for the combinations of each monoterpen and simvastatin [28].

In conclusion, our study shows that linalool and 1,8-cineole are non-toxic monoterpenes that inhibit A549 cell growth through cell cycle arrest but not apoptosis. Oxidative stress-induced by linalool plays a role in the mitochondrial membrane potential depolarization and inhibition of cell proliferation. Both monoterpenes inhibited cell migration. Moreover, linalool and 1,8-cineole sensitized A549 cells to simvastatin-triggered cell cycle arrest and apoptosis. Further investigation is required to disclose whether alternative ways of non-apoptotic cancer cell death or senescence are involved, as well as to explore additional anti-metastatic mechanisms and signaling pathways implicated. Although in vivo experiments are needed to confirm the in vitro results, these findings suggest that linalool and 1,8-cineole have potential as cytostatic, anti-metastatic, and chemosensitizer compounds in NSCLC therapy.

Declarations

Author contribution statement

B. Rodenak-Kladniew: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

M. Agustina Castro and M. Galle: Performed the experiments; Contributed reagents, materials, analysis tools or data.

R. Crespo: Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data.

M. García de Bravo: Conceived and designed the experiments; Contributed reagents, materials, analysis tools or data.

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Data availability statement

Data will be made available on request.

Declaration of interests statement

The authors declare no conflict of interest.
Additional information

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