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Synergistic antiproliferative and anticholesterogenic effects of linalool, 1,8-cineole, and simvastatin on human cell lines

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ABSTRACT

Monoterpenes are naturally occurring plant hydrocarbons with multiple effects on the mevalonate pathway (MP), while statins competitively inhibit hydroxymethylglutarylcoenzyme-A reductase (HMGCR), the rate-limiting enzyme in the MP. Monoterpenes and statins proved capable of inhibiting both proliferation and cholesterogenesis. In the present study we assess the *in vitro* antiproliferative and anticholesterogenic effects of two monoterpenes: linalool and 1,8-cineole—either alone, in combination with each other, or combined individually with simvastatin—on liver-derived (HepG2) and extrahepatic (A549) cell lines. The three compounds alone inhibited cell proliferation in a dose-dependent fashion, while their pairwise combination produced synergistic antiproliferative effects in both cell lines. Incorporation experiments with [^{14}C]acetate revealed that linalool and 1,8-cineole inhibited the MP, probably at different points, resulting in a reduction in cholesterogenesis and an accumulation of other MP intermediates and products. Linalool or 1,8-cineole, either together or individually with simvastatin, synergistically inhibited cholesterol synthesis. At low concentrations both monoterpenes inhibited steps specifically involved in cholesterol synthesis, whereas at higher concentrations HMGCR levels became down-regulated. Added exogenous mevalonate failed to reverse the inhibition of proliferation exerted by linalool and 1,8-cineole, suggesting that HMGCR inhibition alone is not responsible for the antiproliferative activity of those agents. This work demonstrates that monoterpenes in combination with each other, or individually in combination with simvastatin synergistically inhibits proliferation and cholesterogenesis in the human cell lines investigated, thus contributing to a clearer understanding of the action of essential-oil components, and their combination with the statins, in the targeting of specific points within a complex metabolic pathway.

1. Introduction

The mevalonate pathway (MP) is a highly branched metabolic sequence that provides cells with bioactive molecules crucial in multiple cellular processes. The end products of the MP include sterol isoprenoids, such as cholesterol, and nonsterol isoprenoids, such as heme-A, dolichol, and ubiquinone [1,2].

The major branch point of this pathway resides at farnesyl diphosphate (FPP), precursor of the different final products. Furthermore, the addition of an isoprene unit to FPP yields geranylgeranyl diphosphate (GGPP). FPP and GGPP can be post-translationally adducted onto regulatory proteins by protein prenyltransferases to enable protein anchoraging to internal cell membranes and a consequent functional activation. Most of the known prenylated proteins are small GTP-binding species, including the Ras family—with those controlling cell growth and proliferation—and the Rho family—those being crucial mediators of cell migration—[3].

The rate-limiting point of the MP is the conversion of 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) to mevalonic acid, a reaction catalyzed by HMG-CoA reductase (HMGCR), one of the most extensively regulated enzymes in nature [2]. This regulation of the MP, however, can occur at multiple levels throughout the pathway [4].

Various drugs capable of interfering with the MP have been developed, among which pharmacons the statins (*e.g.*, lovastatin, simvastatin, and atorvastatin) competitively inhibit HMGCR [5,6] and deplete cells of downstream isoprenoids, including FPP and GGPP [7]; thus resulting in a reduction in FPP and a decreased *de novo* cholesterol synthesis. Although the statins are used abundantly and effectively in the treatment of hypercholesterolemia, side effects are associated with their use, such as myopathy and hepatotoxicity [8].

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Statins also exhibit antitumor activities in culture against various tumor cells of different origin, which action results primarily from a suppression of proliferation and an induction of apoptosis [9,10]. Nevertheless, conflicting results have been reported on the application of statins as anticancer agents in clinical practice [11–13]. The high concentrations needed to inhibit cell proliferation have been associated with an elevated toxicity, thus restricting their use as a monotherapeutic agent for cancer treatment.

In contrast, monoterpenes—components of the essential oils of many plants including herbs, vegetables, and fruits—are naturally occurring hydrocarbons produced by the condensation of two isoprene units that are used as raw materials in many fields; including spices, phytotherapy, perfumes, and cosmetics [14].

Since monoterpenes are relatively nontoxic, inexpensive, and available in an ingestive form; an increasing interest has arisen in the potential use of essential oils for treating different pathologies of relevant social impact such as diabetes [15], cancer [16], and hypercholesterolemia [17,18]. Certain monoterpenes and essential oils exhibit anticholesterogenic, antiproliferative, and proapoptotic activities in culture as well as moderate hypocholesterolemic, chemopreventive, and chemotherapeutic actions *in vivo* [16–21] These compounds have been suggested as exerting their action through multiple effects on the MP, including an inhibition of protein prenylation and a noncompetitive suppression of HMGCR activity [20,22] along with the targeting of certain other loci specifically involved in cholesterol synthesis [23,24].

Given that the mechanisms of action by which monoterpenes and statins inhibit the MP are different, we hypothesized that a combined treatment between one or more monoterpene and a statin might exert synergistic antiproliferative and anticholesterogenic effects.

Simvastatin, a compound derived synthetically from a fermentation product of *Aspergillus terreus*, is one of the most widely used statins in the treatment of hypercholesterolemia because of the ability to decrease cholesterol synthesis by acting primarily at the hepatic level.

Linalool is a naturally occurring monoterpene alcohol with a pleasant scent present in more than 200 species of plants such as mint, laurel, cinnamon, and citrus fruits and is also either a major or usual compound in most herbal essential oils and in both green and black teas [25].

The cyclic monoterpene oxide 1,8-cineole (cineole, eucalyptol) is present in many essential oils of plants including the eucalyptus and is traditionally used as a food flavoring agent, for treating symptoms of airway diseases, and in aromatherapy [26].

The present study was designed to determine the action of linalool and 1,8-cineole—both in combination or one of the two along with simvastatin—on cholesterogenesis and cellular proliferation in the liver-derived (HepG2) and extrahepatic (A549) tumor cells in culture.

2. Materials and methods

2.1. Reagents

Solvents were obtained from Carlo Erba (Milan, Italy); linalool >95%, 1,8-cineole 99%, mevalonolactone 97%, and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) from Sigma–Aldrich (St. Louis, MO, USA); neutral red from Anedra (Argentina); [^{14}C]acetate (56.8 Ci/mol) from Perkin Elmer Life Science, Inc. (Boston, MA); streptomycin Richet (Argentina); while Merck, Sharp and Dohme (Argentina) kindly provided simvastatin. The sodium salt of simvastatin was prepared by dissolving the drug in ethanol at 60 °C, adding equimolar amounts of NaOH, and then incubating at 60 °C for 1 h. The ethanol was finally evaporated under a stream of nitrogen and the salt dissolved in distilled water at a final concentration of 10 mg/ml [19].

2.2. Cell culture and treatment

The HepG2 human-hepatoma cells were purchased from the American Type Culture Collection. The A549 human-alveolar-adenocarcinoma cells were kindly provided by Dr. Amada Segal-Eiras (CINIBA, UNLP. Argentina). The cells were maintained in 75-cm^2 flasks in filter-sterilized Eagle's Minimal Essential Medium (MEM; Gibco, Invitrogen Corporation) supplemented with (Natocor, Córdoba, Argentina) 10% (v/v) fetal-bovine serum plus 0.1 mg.l^{-1} streptomycin in a humidified incubator at 5% (v/v) CO_2/air and 37 °C.

Cells were grown under standard conditions for 48 h. The medium was changed to fresh MEM plus 10% fetal-bovine serum containing increasing concentrations of linalool, 1,8-cineole, or simvastatin (to determine the half maximal inhibitory concentrations—the IC_{50}—for cell growth and cholesterol biosynthesis) or a combination of those compounds in pairs (to evaluate possible synergistic effects). For the structures of the two monoterpenes and the statin cf. Fig. 1. After 24 h, the cells were washed twice with phosphate-buffered saline (PBS: NaCl 137 mM; KCl 2.7 mM, 10.0 mM Na_2HPO_4, 2.0 mM KH_2PO_4, pH 7.4) and incubated in serum-free MEM Zinc option (IMEM-Zo) with the same additions for another 24 h.

In experiments with mevalonate, cells were treated with linalool, 1,8-cineole, or simvastatin plus mevalonate for 48 h under the same conditions as described for the monoterpenes and the statin.

The linalool, 1,8-cineole, and simvastatin added to the media had been previously dissolved in dimethyl sulfoxide. The final concentration of that vehicle in the control and supplemented media was 0.2% (v/v).

2.3. Cell viability and cell proliferation

2.3.1. MTT assay

Cell viability was measured by the MTT assay [27]. Since the cell lines employed in the present study had different proliferation rates, the number of cultured cells was adjusted to a density such that the cells grew exponentially before initiating the experimental incubations and that insured at the same time a linear relationship between cell number and the optical density as measured in the MTT assay at end of all treatments—that is, the HepG2 and A549 cells were seeded in 24-well plates at densities of 1.5×10^4 and 0.75×10^4 cells per well, respectively.

Cells were treated as described in Section 2.2 and incubated with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) at 0.5 mg/ml in PBS for 3 h. The resulting formazan was dissolved in 0.04 M HCl in isopropanol and the absorbance at 560 nm measured with an Elisa reader (Beckman Coulter DTX 880 Multimode Detector).

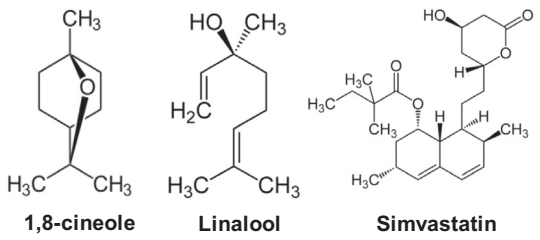

Fig. 1. Structural formulas of 1,8-cineole, linalool, and simvastatin.

2.3.2. Neutral-red assay

The viability of the HepG2 and A549 cells was also assessed through the use of the neutral-red (NR) incorporation assay. Cells were seeded and treated as described for the MTT method. After treatment, the medium was replaced with 0.5 ml per well of NR solution (0.05 mg/ml in serum-free IMEMZo) and the plates incubated for 3 h at 37 °C. The cells were rinsed twice with PBS and 0.75 ml/well of stain-extraction solution (50% [v/v] ethanol in 2% [v/v] aqueous acetic acid|) added. The plates were shaken for 15 min at 50 rpm and the absorbance read at 540 nm [28].

2.3.3. Cell counting

Cells were seeded and treated as described for the MTT and NR methods. After treatment, the cells were harvested by trypsinization and the cell suspensions mixed (1/1, v/v) with 0.4% (w/v) trypan-blue solution. Proliferation was determined by counting trypan-blue–excluding cells in a Neubauer hemocytometer.

2.4. Incorporation of [^{14}C]acetate

HepG2 and A549 cells were seeded in 6-well plates at respective densities of 1.5×10^5 and 1×10^5 per well and treated as described previously with the addition of [^{14}C]acetate (2 μCi/ml culture medium) over the final 3 h. The cells were then washed three times in PBS, the lipids extracted with 3 ml *n*-hexane/isopropanol 3/2 (v/v) for 30 min, and the cell pellet dissolved in 2 ml of 0.1 M NaOH for protein determination [29]. The lipid extract was transferred to a glass tube, evaporated to dryness under a stream of nitrogen, resuspended in 1.5 ml of 10% (v/v) KOH in methanol, and saponified at 80 °C for 45 min. The nonsaponifiable lipids were extracted with hexane and an aliquot used to determine the radioactivity of the samples by liquid-scintillation counting in a Wallac 1214 RackBeta counter (Pharmacia, Turku, Finland).

Cholesterol and other MP metabolites from the nonsaponifiable fraction were separated by thin-layer chromatography on silica gel G after development in 100% chloroform and visualized by autoradiography in a Storage Phosphor Screen, GE Healthcare. Quantitative densitometric analyses were performed by means of the Image J program. All lipid classes were identified by comparison with a standard mixture containing cholesterol, lanosterol, dolichol, ubiquinone, and squalene added to the same plate.

2.5. Free- and esterified-cholesterol content

HepG2 and A549 cells were seeded in culture flasks and treated as described above. The total lipids were extracted from the cell pellets with methanol/chloroform 2/1 (v/v) [30]. Free and esterified cholesterol were separated by thin-layer chromatography on silica gel G developed in hexane/diethylether/acetic acid 80/20/1 (v/v/v), revealed through the use of an acidic ferric-chloride solution as a spray reagent [24], and quantified by means of a curve constructed with pure standards that had been run on the same plate. The spot images were analyzed by the Image J program.

2.6. Western blotting

HepG2 cells were seeded in 25-cm^2 flasks at a density of 5×10^5 per flask and treated as described in Section 2. Cells were harvested by scraping in PBS and resuspended by vigorous pipetting in 5 ml of the same solution. After removing an aliquot to determine cellular protein content [29], the cells were collected by centrifugation. The cell pellet was resuspended in 2× Laemmli lysis buffer by vortexing and boiled for 5 min. The chromatin was sheared by passage through a 26-gauge needle. The cell lysates (100 μg of cellular protein) were resolved on 12.5% (w/v) sodiumdodecylsulfide–polyacrylamide gels (Amersham, GE Healthcare) then migrated onto polyvinylidene–difluoride membranes by semidry transfer at 10 V for 60 min. The membrane was blocked with 5% (w/v) nonfat dry milk in PBS (pH 7.4) containing 0.1% (v/v) Tween 20 (PBST) plus 5% (v/v) skimmed milk then incubated with rabbit anti-HMGCR (Santa Cruz, CA, USA) diluted 1/200 in antibody-dilution buffer (2% [v/v] skimmed milk in PBST) for 2 h at room temperature. After three 5-min washes in PBST, horseradish-peroxidase-conjugated goat anti(rabbit IgG) antibodies (Thermo Scientific) diluted 1/3000 in antibody-dilution buffer were added to the membrane for 1 h. Immunoreactive bands were detected by enhanced-chemiluminescence Western-blot–detection reagents (Amersham Pharmacia Biotech) and processed through the use of common X-ray-film developers and fixers. The band intensity was quantified by Image J software.

2.7. Analysis of drug synergism

The analysis of drug synergism was performed according to the method of Kern et al. as applied to a measurement of the proliferation of viable cells and the inhibition of cholesterogenesis [31].

For this purpose, a synergistic ratio: $R = (\text{Pexp})/(\text{Pobs})$ was calculated, where:

Pexp corresponds to the expected value of the process under consideration (i.e., proliferation or cholesterogenesis) calculated as the numerical product of the percentage remaining after treatment with drug X alone times the percentage remaining after treatment with drug Y alone divided by 100.
Pobs corresponds to the actual percent of the process remaining under the influence of X and Y in combination.

Synergy was defined as any value of $R > 1$, while an $R = 1.0$ (additive effect) or less indicated the absence of a synergistic interaction.

For example, if in the presence of the individual drugs X and Y the respective proliferations were 75% and 50% while the value observed for the two drugs in combination were 30%, R would be $(75)(50)/(100)(30) = 37.5/30 = 1.25$, indicating the occurrence of synergism.

2.8. Statistical analysis

Experimental data are expressed as the means ± SD. Statistical analysis was performed through the use of the one-way analysis of variance (ANOVA) and the Tukey–Kramer multiple-comparisons test with the significance level set at $p < 0.05$ or the unpaired *t*-test (GraphPad inStat program). The IC_{50} values of linalool, 1,8-cineole, or simvastatin for cell proliferation (IC_{50}) or cholesterol synthesis ($IC_{50}CS$) were calculated by nonlinear-regression curves (SigmaPlot software; Systat Software, Inc., Point Richmond, CA).

3. Results

3.1. Effect of linalool, 1,8-cineole, and simvastatin, either alone or in pairwise combination, on cell viability and proliferation

In order to evaluate the antiproliferative activity of linalool, 1,8-cineole, or simvastatin exponentially growing cells were incubated with increasing amounts of those compounds and cell proliferation measured by cell counting. Treatment of HepG2 and A549 cells with up to 2500 and 2000 μM linalool, respectively, produced a dose-dependent inhibition of cell proliferation (Fig. 2 panels A and B). Likewise, exposure to 1,8-cineole caused a growth inhibition in a dose-dependent manner within the range of 4000–8000 μM in HepG2 and of 2400–10000 μM in A549 cells (Fig. 2, panels C and D). Finally, cells incubated with simvastatin

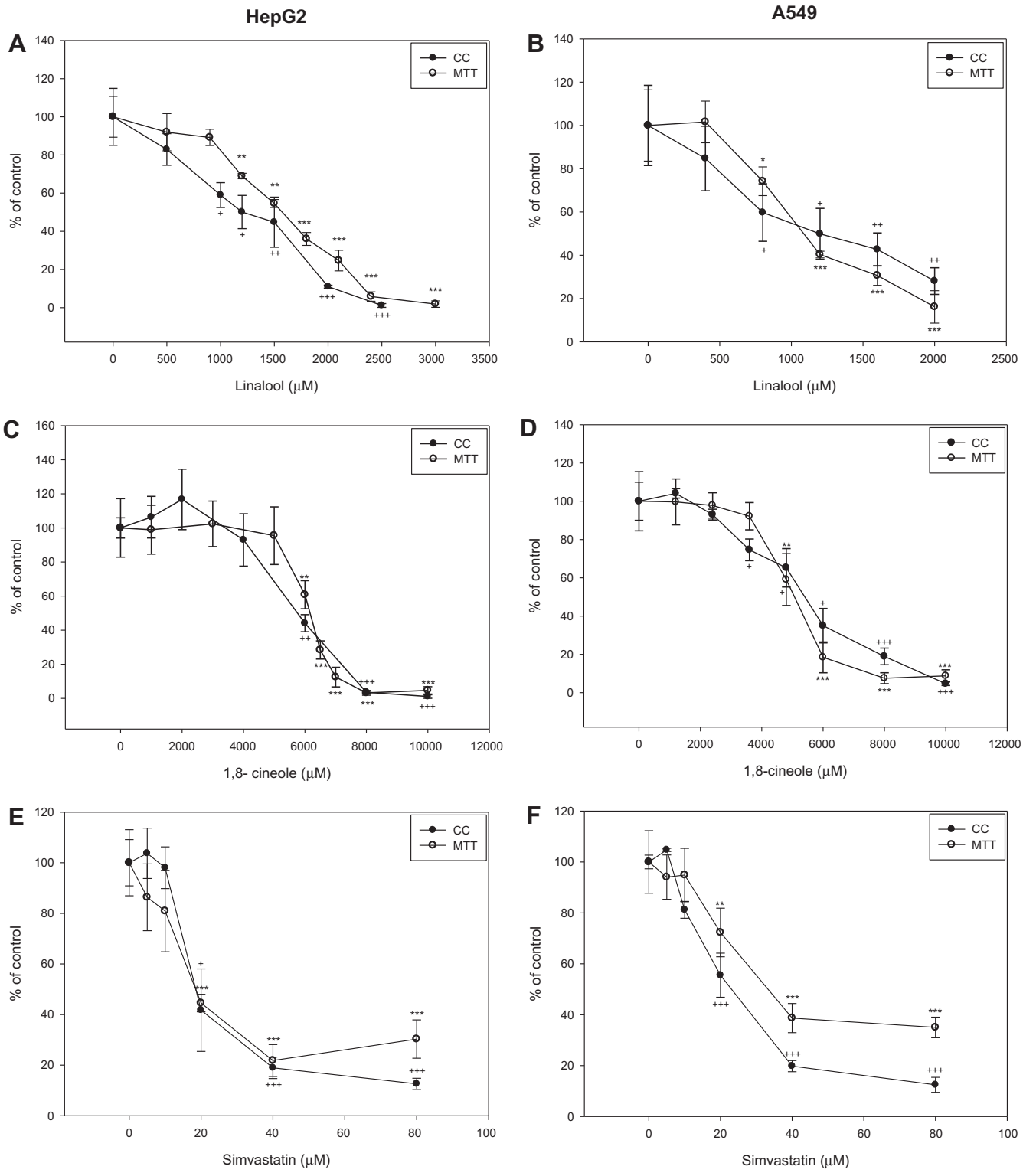

Fig. 2. Effect of linalool, 1,8-cineole, and simvastatin on cell viability and proliferation in HepG2 and A549 cells. The cultured cells in exponential growth were treated with increasing concentrations of each compound for 48 h. Cell viability and proliferation were determined by the MTT assay and cell counting (CC), respectively, in the HepG2 (A, C, and E) and A549 (B, D, and F) cells. Data are expressed as the means ± SD of three independent experiments performed in at least triplicate; $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

exhibited a dose-dependent growth inhibition—from 10 to 40 μM of the drug in both cell lines. Thereafter the inhibition curve plateaued up to 80 μM, the maximum concentration tested (Fig. 2, panels E and F). Similar results to those for cell counting were obtained for the viability assays MTT (Fig. 2) and NR (curves not shown). Table 1 shows the IC_{50} values estimated graphically for the three different methods.

We evaluated the ability of exogenous mevalonate to restore cell growth in order to define if the effects of the monoterpenes on cell proliferation and HMGCR were causally related. Mevalonate addition to the culture medium did not affect cell proliferation at any concentration of linalool and 1,8-cineole up to approximately their IC_{50} value (Table 2). Exogenous mevalonate, as expected, significantly restored cell growth in simvastatin-treated cells.

Table 1
Antiproliferative activity of linalool, 1,8-cineole and simvastatin on HepG2 and A549 cells.

	HepG2			A549		
	IC_{50} (μM)			IC_{50} (μM)		
	CN	MTT	NR	CN	MTT	NR
Linalool	1191 ± 156	1550 ± 98	1600 ± 70	1160 ± 133	1093 ± 41	1750 ± 190
1,8-Cineole	5773 ± 63	6050 ± 163	8180 ± 110	5370 ± 186	5030 ± 203	7110 ± 240
Simvastatin	18.9 ± 1.9	18.2 ± 1.1	23.0 ± 1.2	21.6 ± 0.8	27.5 ± 1.9	19.2 ± 1.2

The cultured cells of both cell lines in exponential growth were treated with increasing concentrations of linalool, 1,8-cineole, and simvastatin for 48 h. Cell proliferation and viability was determined by counting cell number (CN), MTT and NR assay. Dose–response curves were obtained by linear and nonlinear regression and the IC50 values calculated. Data are means ± SD. Each experiment was carried out in triplicate.

To analyze the potential synergistic effect of pairwise combinations of linalool, 1,8-cineole, and simvastatin; cells were incubated with concentrations of each of those compounds alone that did not inhibit cell proliferation significantly, while parallel cultures were tested in the same way but with the pairs of inhibitors at the same respective concentrations. Cell viability and proliferation were determined by the MTT and NR assays and by cell counting, respectively. The two monoterpenes in combination or one of those combined with simvastatin significantly inhibited cell viability and proliferation, and at *R* values greater than unity indicating a synergistic interaction (Fig. 3).

In order to ratify the synergistic antiproliferative effects of these agents, other combinations at different concentrations were tested and the MTT assay performed. Most of the combinations resulted in a clear synergistic interaction between the monoterpenes and between each monoterpene and simvastatin (Table 3).

3.2. Incorporation of [^{14}C]acetate into nonsaponifiable lipids

Quantification by the incorporation of radioactivity was used to determine the extent to which [^{14}C]acetate was metabolized to cholesterol and other nonsaponifiable lipids (*e.g.*, lanosterol, squalene, and ubiquinone) after incubating the cells with increasing concentrations of each compound (Fig. 4). Linalool, 1,8-cineole, and simvastatin significantly suppressed radioactivity incorporation into cholesterol at 100 μM, 250 μM, and 0.1 μM in HepG2 cells and at 200 μM, 250 μM, and 1 μM in A549 cells, respectively. Table 4 summarizes the resulting IC_{50}CS values.

In the HepG2 cells, the incorporation of acetate into squalene and lanosterol (MP intermediates specifically involved in cholesterol synthesis) and into ubiquinone (|a separate|final product of the MP) could be quantified for all treatments.

Linalool concentrations that inhibited cholesterogenesis by about 50% (400–600 μM) increased radioactivity incorporation into lanosterol and ubiquinone, whereas the incorporation into squalene was either unchanged or slightly diminished (Fig. 4, Panel A). In contrast, in HepG2 cells at concentrations of 1,8-cineole that inhibited *de novo* cholesterogenesis by between one-half and three-quarters (500–1000 μM) the labeling from acetate decreased in lanosterol and increased in ubiquinone, but remained essentially unchanged in squalene (Fig. 4, Panel C). From that higher concentration of 1,8-cineole upward, the incorporation into lanosterol, squalene, and ubiquinone dropped markedly at 2000 μM and drastically so when cholesterogenesis was inhibited by over 90% at 3000 μM (Fig. 4, Panel C).

Simvastatin concentrations that significantly inhibited cholesterol synthesis also decreased acetate incorporation into both squalene and lanosterol as well as into ubiquinone (Fig. 4, Panel E).

In the A549 cells, only the incorporation of [^{14}C]acetate into cholesterol and total nonsaponifiable lipids could be quantified (Fig. 4, panels B, D, and F), while MP intermediates and final products of the pathway remained undetectable under all treatment conditions. When cells were treated with the monoterpenes, the incorporation into cholesterol decreased to a greater extent than into nonsaponifiable lipids suggesting a redistribution into other MP intermediates and/or ubiquinone. Even when the incorporation of labelled acetate into ubiquinone could not be quantified in all experiments, an increased value was found in the linalool- and 1,8-cineole-treated cells, consistent with the results obtained for the HepG2 cells (data not shown).

To evaluate potential synergistic effects in the inhibition of cholesterol synthesis, cells were treated with pairwise combinations of these compounds at low concentrations (corresponding to approximately 1/3–1/2 of the IC_{50}CS values). All the pairings resulted in synergistic effects on the HepG2 cells at *R* values greater than 1. In contrast, in the A549 cells the combination of 1,8-cineole and simvastatin—unlike the other two pairings—inhibited only at an *R* of <1 (Fig. 5).

3.3. Free- and esterified-cholesterol content

HepG2 and A549 cells were exposed to the same concentrations and combinations of linalool, 1,8-cineole, and simvastatin used in the assays testing synergism in the inhibition of cholesterogenesis, and then the cellular content of free and esterified cholesterol was determined (Fig. 6). Each compound alone did not produce a

Table 2
Failure to restore cell growth in HepG2 and A549 cells inhibited by linalool and 1,8-cineole through the addition of exogenous mevalonate.

	HepG2			A549	
	−Mv	+Mv		−Mv	+Mv
Control	100 ± 7.7	106.4 ± 14.7	Control	100 ± 12.8	102.7 ± 13.6
Ln 1000 μM	94.3 ± 10.9	84.0 ± 13.0	Ln 800 μM	73.7 ± 5.3	62.9 ± 7.3
Ln 1500 μM	62.1 ± 11.2	67.8 ± 9.0	Ln 1200 μM	55.5 ± 6.1	61.2 ± 3.4
Cn 4000 μM	98.2 ± 13.6	101.7 ± 9.8	Cn 3000 μM	86.3 ± 10.1	81.0 ± 12.1
Cn 6000 μM	56.0 ± 13.6	54.9 ± 9.5	Cn 5000 μM	58.2 ± 2.5	64.3 ± 11.3
Sv 20 μM	41.4 ± 6.5	114.0 ± 19.1***	Sv 25 μM	62.1 ± 5.2	79.4 ± 8.3*

HepG2 and A549 in exponential growth in 24-well plates were treated with increasing concentrations of linalool (Ln), 1,8-cineole (Cn), or simvastatin (Sv) with or without mevalonate (Mv) (0.5 mM) for 48 h. Cell proliferation and viability was determined by the MTT assay. Data, expressed as a percent of the control, are the mean ± SD of three independent experiments repeated in quadruplicate. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ vs. the same treatment without Mv.

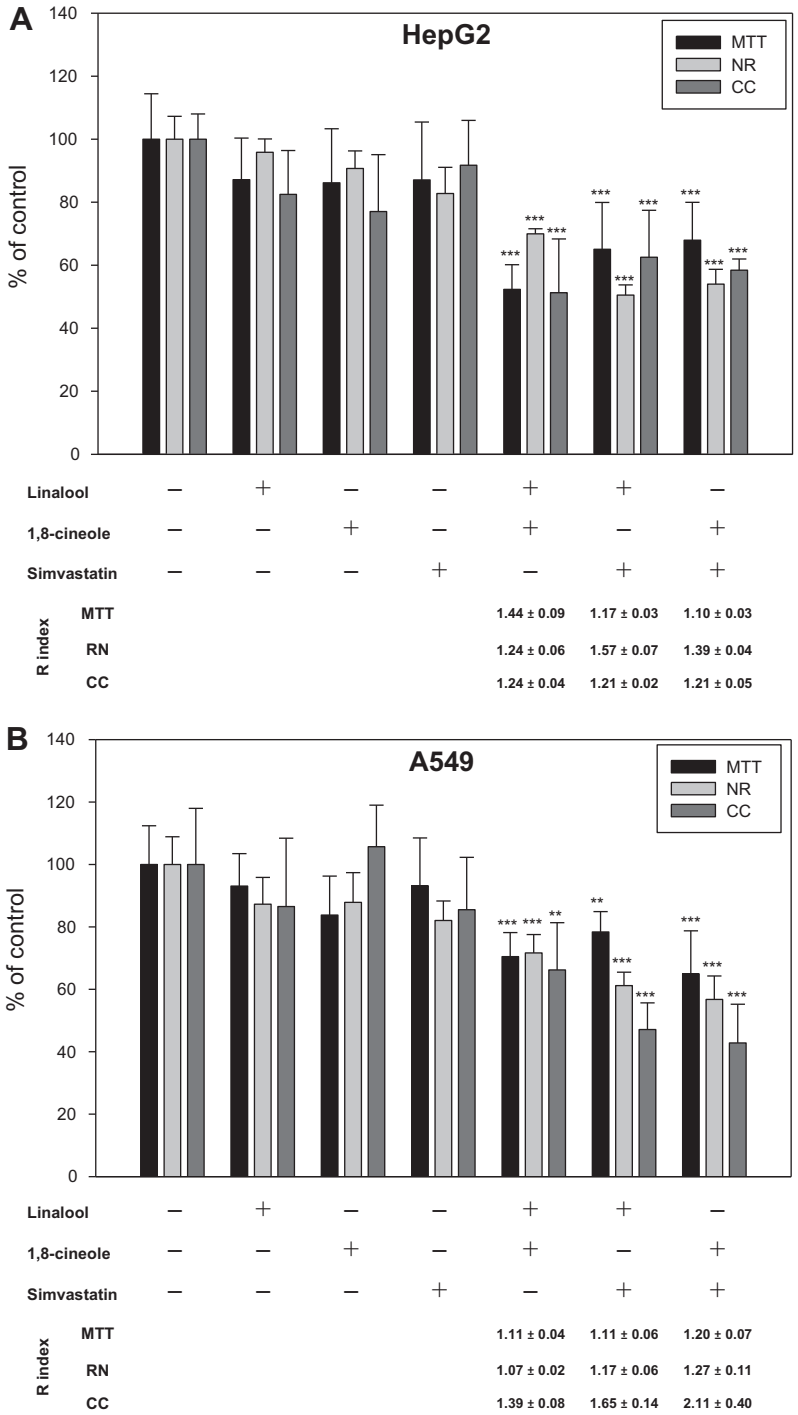

Fig. 3. Effects of the combinations of linalool, 1,8-cineole, and simvastatin on HepG2 (A) and A549 (B) viability and proliferation. The cultured cells in exponential growth were treated with the monoterpenes at one-half their respective IC50 concentrations and with simvastatin at 5 μM (HepG2) or 10 μM (A549), either alone or in pairwise combination, for 48 h, and cell viability and proliferation were determined by the MTT and NR assays and by cell counting (CC), respectively. Synergistic effects were evaluated by the *R* index as described in Section 2. An $R > 1$ indicates synergism, while an $R = 1.0$ (additive effect) or less denotes an absence of synergism. Data are expressed as the means ± SD from three independent experiments carried out in at least triplicate; $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

significant decrease in cholesterol levels in these cell lines (Fig. 6). Contrary to expectations, the A549 cells treated with 200 μM linalool developed an increased cholesterol content that became normalized when the cells were coincubated with either 1,8-cineole or simvastatin (Fig. 6, Panel B). Whereas no significant differences were obtained when either cell line was treated with linalool and 1,8-cineole in combination, the presence of either monoterpene plus simvastatin produced a reduction in cholesterol content in the HepG2 cells, though not in the A549 line.

3.4. HMGCR levels

Western-blot analysis of the HMGCR in HepG2 cells treated with linalool at 200 μM or 1,8-cineole at 750 μM—concentrations that inhibit cholesterol synthesis significantly but not acetate incorporation into nonsaponifiable lipids—indicated that HMGCR levels remained similar to those obtained for control cells. A combined treatment at these concentrations produced a slight decrease (*i.e.*, 20%) in HMGCR levels (Fig. 7). When, however, the cells were

Table 3
Multiple pairwise combinations of linalool, 1,8-cineole and simvastatin at different concentrations.

HepG2											
Linalool	500 μM	+	+	+	+	–	–	–	–	–	–
	1000 μM	–	–	–	–	+	+	+	+	–	–
1,8-Cineole	2000 μM	+	–	–	–	+	–	–	–	+	–
	4000 μM	–	+	–	–	–	+	–	–	–	+
Simvastatin	5 μM	–	–	+	–	–	–	+	–	+	+
	10 μM	–	–	–	+	–	–	–	+	–	–
R index		1.91 ± 0.30	1.57 ± 0.04	1.15 ± 0.08	1.48 ± 0.19	2.46 ± 0.91	3.62 ± 0.84	1.39 ± 0.09	2.11 ± 0.04	1.03 ± 0.11	1.22 ± 0.12
A549											
Linalool	400 μM	+	+	+	+	–	–	–	–	–	–
	800 μM	–	–	–	–	+	+	+	+	–	–
1,8-Cineole	2000 μM	+	–	–	–	+	–	–	–	+	–
	3000 μM	–	+	–	–	–	+	–	–	–	+
Simvastatin	5 μM	–	–	+	–	–	–	+	–	+	+
	10 μM	–	–	–	+	–	–	–	+	–	–
R index		1.46 ± 0.05	2.06 ± 0.09	1.57 ± 0.09	1.76 ± 0.21	1.72 ± 0.33	1.99 ± 0.18	1.65 ± 0.11	2.47 ± 0.43	1.00 ± 0.04	1.30 ± 0.01

The cultured cells of both cell lines in exponential growth were treated with multiple combinations in pairs of the three compounds at different concentrations. Cell proliferation and viability was determined by the MTT assay. Each experiment was carried out in triplicate and repeated in three independent experiments. Data expressed as the mean ± SD.

treated with linalool at 800 μM or 1,8-cineole at 3000 μM—concentrations that significantly inhibit both cholesterol synthesis and acetate incorporation into nonsaponifiable lipids—the content of HMGCR dropped notably, with the inhibition being significantly higher than that observed for the lower concentration of each respective monoterpene (Fig. 7). Simvastatin at 5 μM was employed as a positive control for increased HMGCR levels.

4. Discussion

We have previously reported that the monoterpene geraniol has multiple effects on lipid metabolism and cell growth and that its combination with simvastatin exerts synergistic antiproliferative and anticholesterogenic effects on the HepG2 cells [22,23,32].

In the present investigation we analyzed the antiproliferative and anticholesterogenic ability of a cyclic (1,8-cineole) and an acyclic (linalool) monoterpene on hepatoblastoma (HepG2) and adenocarcinoma (A549) tumor cells, evaluating the additive or synergistic effects of combinations of the two monoterpenes or of each one alone with simvastatin. To that end we also generated data on the effects of those inhibitors on the MP by monitoring the levels of the HMGCR protein and the incorporation of acetate into nonsaponifiable lipids.

Within this experimental design 0.3 μM simvastatin was required to obtain a 50% inhibition of cholesterogenesis in the liver-derived cells (HepG2). This concentration is similar to those reported by other authors [33] as well as those achieved in plasma during treatment of hypercholesterolemia [34]. Since the MP is inhibited at an early stage, a decrease in cholesterol synthesis is accompanied by a reduced incorporation of [^{14}C]acetate into cholesterogenesis-specific intermediates—*e.g.*, lanosterol and squalene—and other end products of the MP, such as ubiquinone, whose deficiency is often associated with the muscle weakness and rhabdomyolysis caused by statin therapy [35]. This diminution in cholesterol and metabolic intermediates of the MP linked to the transcriptional and posttranscriptional regulation of HMGCR results in increased levels of this enzyme—as reported by many authors [36] and observed by us when HepG2 cells were treated with 5 μM simvastatin—causing a reduction in the effectiveness of the statins. These drugs are thus seen to induce a form of statin tolerance in their target cells. Seven to ten times higher concentrations of simvastatin are required to obtain a 50% inhibition of cholesterol synthesis in A549 cells. This phenomenon could be attributed to the lower cholesterogenic activity of extrahepatic cells and/or the hepatoselectivity of this statin. By contrast, linalool and 1,8-cineole exert anticholesterogenic effects in a nonhepatoselective way, with IC_{50}CS values within the same order of magnitude in both cell lines. The inhibition of cholesterol synthesis with these monoterpenes appears to be attributable to multiple effects on the MP. Here we demonstrated that concentrations of the two compounds that inhibit cholesterogenesis by about 50% (200 μM linalool and 750 μM 1,8-cineole) also block other stages in the MP without, however, altering HMGCR levels. The acyclic monoterpene linalool would exert its action by inhibiting enzyme/s involved in the conversion of lanosterol to cholesterol, while the cyclic isoprenoid 1,8-cineole seems to inhibit enzyme/s mediating the metabolism of squalene to lanosterol. Further data would be required to elucidate which enzyme(s) within the two segments of the MP is/are inhibited by these monoterpenes at low concentrations. In both instances, this inhibition results in a redirection of FPP (metabolite at the main branch point of the MP) into the production of ubiquinone. Similar effects have been reported for other monoterpenes such as geraniol in HepG2 [23] and perillyl alcohol in NIH 3T3 cells [24]. Higher concentrations of monoterpenes (800 μM linalool and 3000 μM 1,8-cineole) decrease HMGCR levels causing a profound inhibition of cholesterogenesis without affecting cell proliferation. In HepG2 cells treated with 500 μM linalool, Cho et al. reported a decrease of 54% in HMGCR levels caused by an inhibition at the transcriptional level along with an increased degradation of the enzyme [37], while we more recently demonstrated that geraniol inhibited HMGCR at a posttranscriptional step [22].

Despite the decrease in the HMGCR levels at high concentrations of the monoterpenes, under our experimental conditions, the inhibition of the conversion of lanosterol into cholesterol was strong enough to cause a detectable accumulation of acetate incorporation into lanosterol and ubiquinone. In contrast, the specific inhibition of the conversion of squalene into lanosterol at high concentrations of 1,8-cineole was apparently insufficient to produce an accumulation of acetate incorporation into the MP intermediates and/or into ubiquinone.

The incorporation of acetate into cholesterol was synergistically inhibited under the combined action of the monoterpenes or a monoterpene with simvastatin. That linalool, 1,8-cineole, and simvastatin at the concentrations used in these experiments inhibit the MP at different points could explain this synergism. When the monoterpenes and simvastatin are combined, the decreased levels of ubiquinone produced by simvastatin seem to be compensated for by the increment observed independently with linalool and 1,8-cineole (data not shown). This *de facto* compensation could

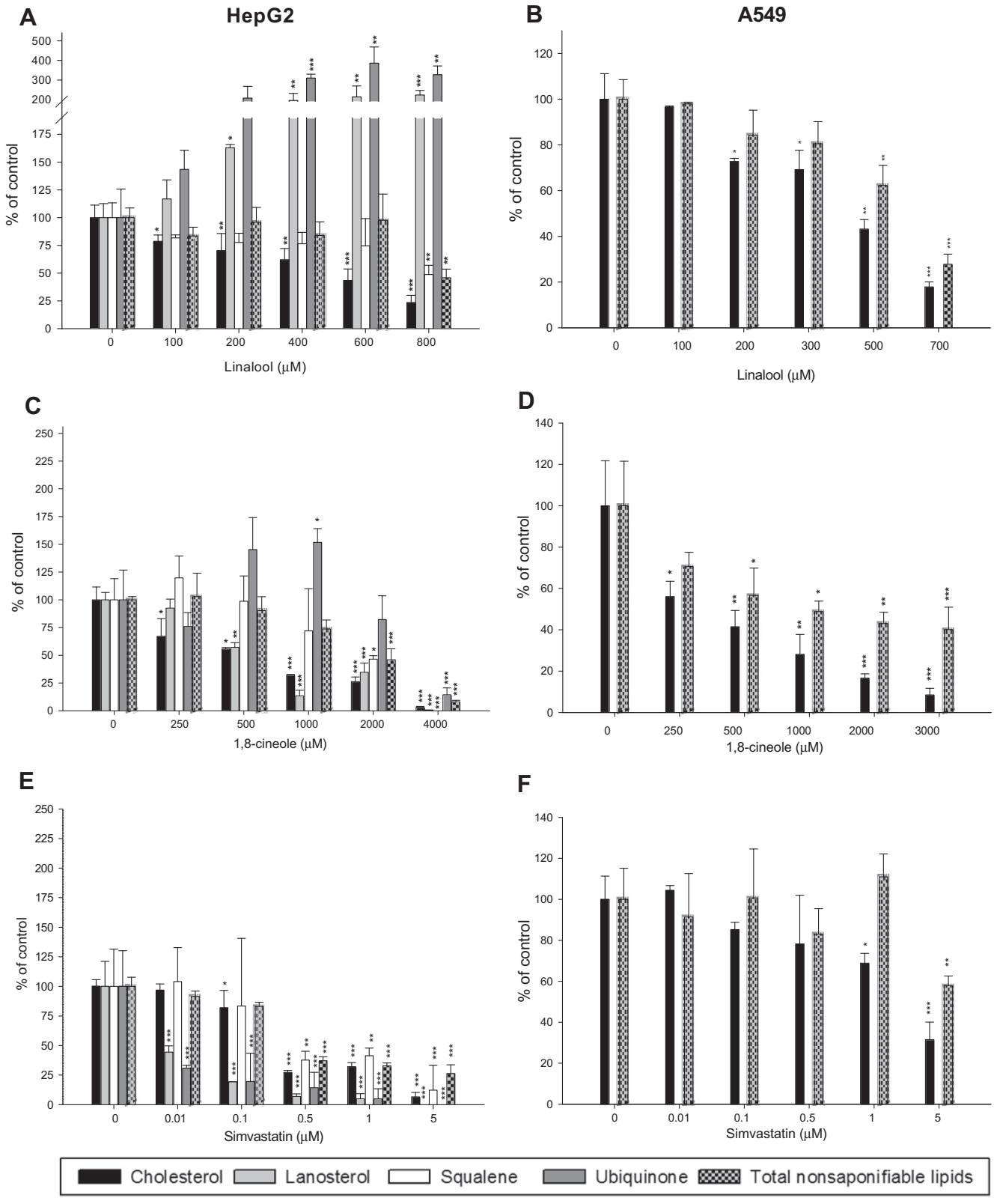

Fig. 4. [^{14}C]acetate incorporation into cholesterol and other nonsaponifiable lipids of the mevalonate pathway in HepG2 and A549 cells. The cultured cells in exponential growth were treated with increasing concentrations of linalool, 1,8-cineole, and simvastatin that do not inhibit cell growth for 48 h, with 2 μCi/ml of [^{14}C]acetate being added over the final 3 h. Radioactivity incorporation into cholesterol, lanosterol, squalene, ubiquinone, and total nonsaponifiable lipids in HepG2 (A, C, and E) and in A549 cells (B, D, and F) was located by autoradiography and quantitative densitometric analyses performed. In A549 cells, lanosterol, squalene, and ubiquinone were undetectable. Data are means ± SD of three independent experiments performed in quadruplicate; $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

Table 4
Anticholesterogenic activity of linalool, 1,8-cineole and simvastatin on HepG2 and A549 cells.

	IC_{50CS} (μM)	
	HepG2	A549
Linalool	490 ± 42	430 ± 29
1,8-Cineole	560 ± 112	345 ± 48
Simvastatin	0.30 ± 0.07	2.10 ± 0.30

Cultured HepG2 and A549 cells in exponential growth were treated with increasing concentrations of linalool, 1,8-cineole, and simvastatin for 48 h. Cholesterol synthesis was quantified by [^{14}C]acetate incorporation into nonsaponifiable lipids. The IC_{50CS} were calculated from dose–response curves obtained by nonlinear regression. Data are means ± SD of three independent experiments, each one carried out in quadruplicate.

prove beneficial within a pharmaceutical context by avoiding the undesirable effects related to the lower levels of ubiquinone caused by higher levels of the statins.

In most instances, under the experimental conditions tested, the inhibition of cholesterogenesis did not produce a decrease in cellular cholesterol levels probably because the cells were able to meet their cholesterol requirement through the uptake of exogenous sterol, as we previously reported for HepG2 cells treated with the monoterpene geraniol [23].

The antiproliferative effects of monoterpenes have been attributed to the inhibition of both protein prenylation—at the level of the prenyl-protein-transferase enzymes—and HMGCR activity in a noncompetitive way [20]. A wide range of linalool and 1,8-cineole concentrations has been used to assess the effects on cell proliferation, apoptosis, or gene expression in HepG2 cells. Mitić-Ćulafić et al. [39] reported that 65 μM linalool or 1,8-cineole did not affect HepG2 cell growth. In addition Cho et al. [37] under conditions that did not inhibit cell proliferation, studied the expression of the sterol-regulatory-element binding protein 2 and HMGCR using 500 μM linalool. Nevertheless, Usta et al. [38] reported that a 50% decrease in the viability of HepG2 cells was achieved with as low as 0.4 μM linalool. Under the conditions used in these experiments linalool and 1,8-cineole inhibited the growth of both cell lines at respective concentrations of 1000 μM and 4000 μM or higher. As with the statins, much higher concentrations of the monoterpenes are required to inhibit cell proliferation than cholesterogenesis, with linalool being the more potent of the two in both cell lines. Exogenous mevalonate was unable to reverse the inhibition of proliferation, suggesting that a mechanism other than impaired HMGCR activity is responsible for the antiproliferative action of linalool and 1,8-cineole, as had been described previously for geraniol [22]. The greater effectiveness of linalool could be related to the powerful ability of this monoterpene to inhibit protein prenylation. Nevertheless, further experiments will be required to confirm this hypothesis.

Many studies have been reported on the effects of different monoterpenes [40,41] and essential oils of various compositions [19,42] on the proliferation of human cells in culture, but to our knowledge no investigations have been undertaken on the combined effects of pure monoterpenes. The results reported here demonstrate that exposure to subeffective doses of linalool and 1,8-cineole produces a synergistic inhibition of proliferation in both HepG2 and A549 cells—*i.e.*, concentrations at half the IC_{50} of each individual compound alone while not inhibiting cell proliferation in a significant manner, in combination produce a significant inhibition of growth in both cell lines, at *R* values greater than unity in all instances. That the synergistic effects between linalool and 1,8-cineole could be confirmed through the use of different combinations (*i.e.*, other concentrations) of these compounds could explain why certain essential oils have more potent effects than exhibited by the principal individual components by themselves. We have already described that mandarin essential oil possesses an antiproliferative activity against HepG2 and A549 cells that is more effective than the inhibition of limonene alone, that oil's principal component [43].

A number of studies have shown that statins can inhibit a wide variety of tumor cells including prostate, gastric, and pancreatic carcinoma, as well as colon adenocarcinoma, neuroblastoma, glioblastoma, mesothelioma, melanoma, and acute myeloid leukemia [44]. In particular simvastatin has been reported to inhibit cell proliferation in concentrations over the range we described here [10,45]. In our experimental design concentrations of simvastatin in the order of 20 μM inhibited cell proliferation in both HepG2 and A549 cells. Taking into account that the maximal prescribed dosage of simvastatin in humans has been associated with multiple toxic side effects [34], we combined simvastatin with

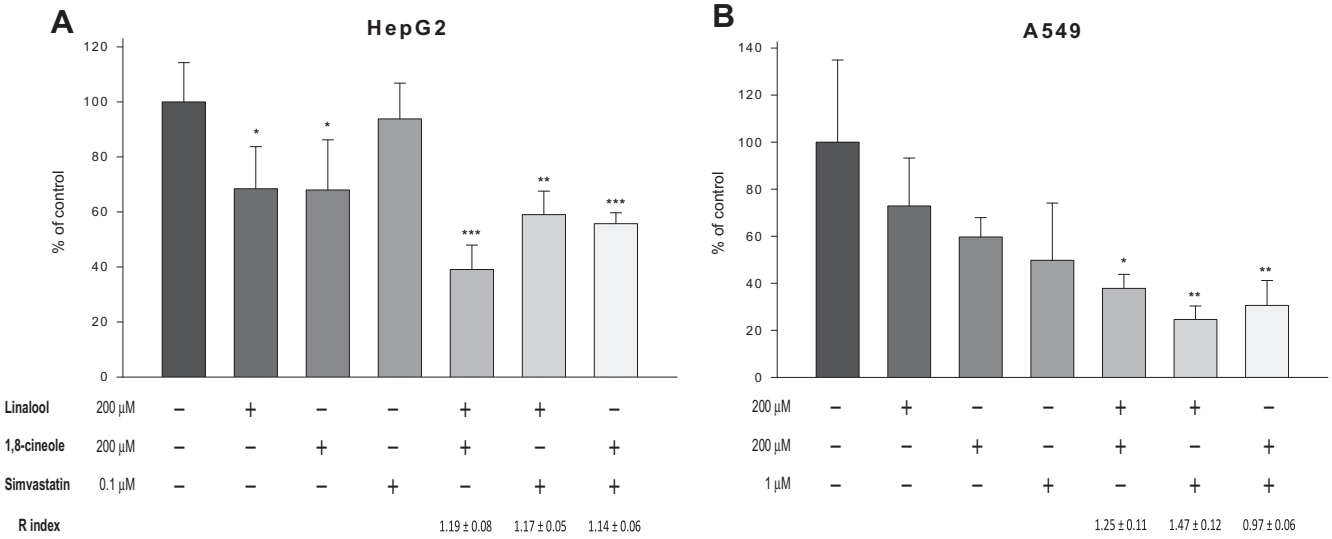

Fig. 5. Effect of the pairwise combinations of linalool, 1,8-cineole, and simvastatin on cholesterol synthesis in HepG2 (A) and A549 (B) cells. The cultured cells in exponential growth were treated with linalool, 1,8-cineole and simvastatin both alone and in pairwise combination for 48 h, with [^{14}C]acetate with 2 μCi/ml being added over the final 3 h. Radioactivity incorporation into cholesterol was located by autoradiography and quantitative densitometric analyses performed. Synergistic effects were evaluated by the *R* index as described in Section 2. $R > 1$ indicates synergism while $R = 1.0$ (additive effect) or less denotes an absence of synergism. Data are means ± SD of three independent experiments, each one carried out in quadruplicate. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

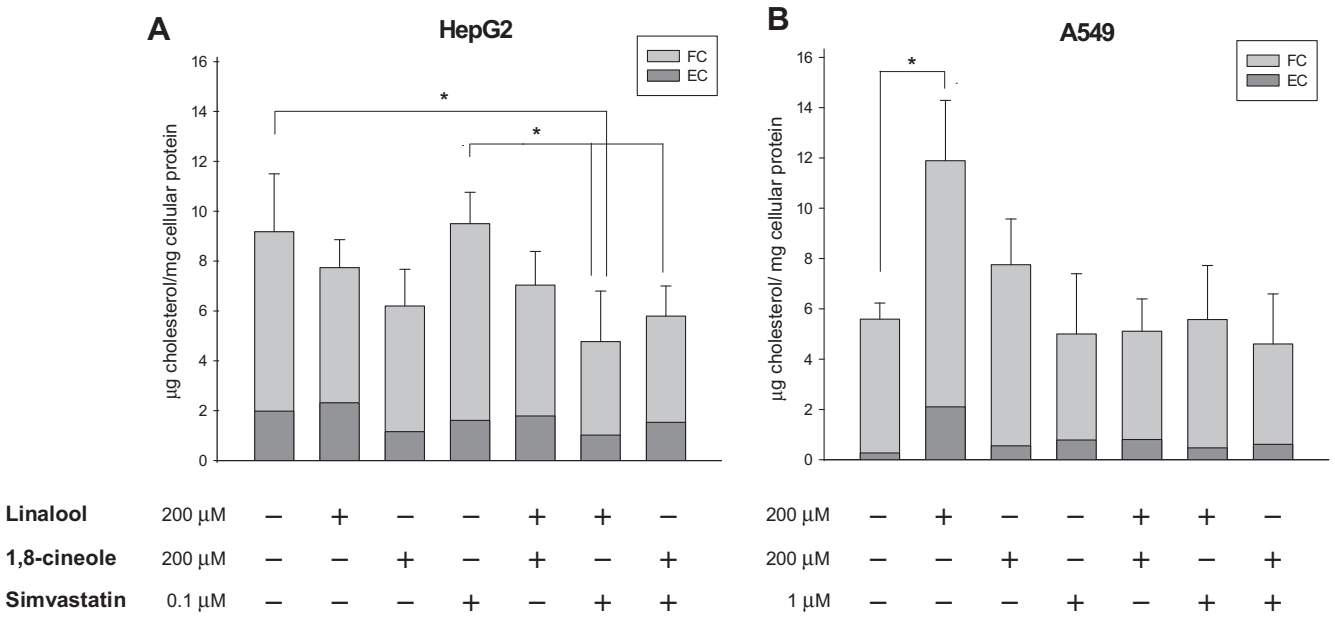

Fig. 6. Effect of the combinations of linalool, 1,8-cineole, and simvastatin on the free and esterified cholesterol content of HepG2 (A) and A549 (B) cells. The cultured cells in exponential growth were treated with linalool, 1,8-cineole, and simvastatin and with pairwise combinations of the three. Free (FC) and esterified (EC) cholesterol were separated by thin-layer chromatography and quantified by densitometric analyses (see Materials and Methods). Data are expressed as the means ± SD of two independent experiments; $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

monoterpenes in order to examine for possible synergistic antiproliferative effects. In a previous publication we had demonstrated that the combination of simvastatin and geraniol, an acyclic monoterpene, synergistically inhibited the proliferation of the HepG2 line [32]. In the present work we therefore combined each isoprenoid at one-half its IC_{50} with subeffective doses of simvastatin with the combinations resulting in *R* values greater than unity (indicating a synergistic inhibition) in both cell lines. Our results may point to a potentially promising approach for future clinical trials that would employ lower, or even subliminal, doses of statins

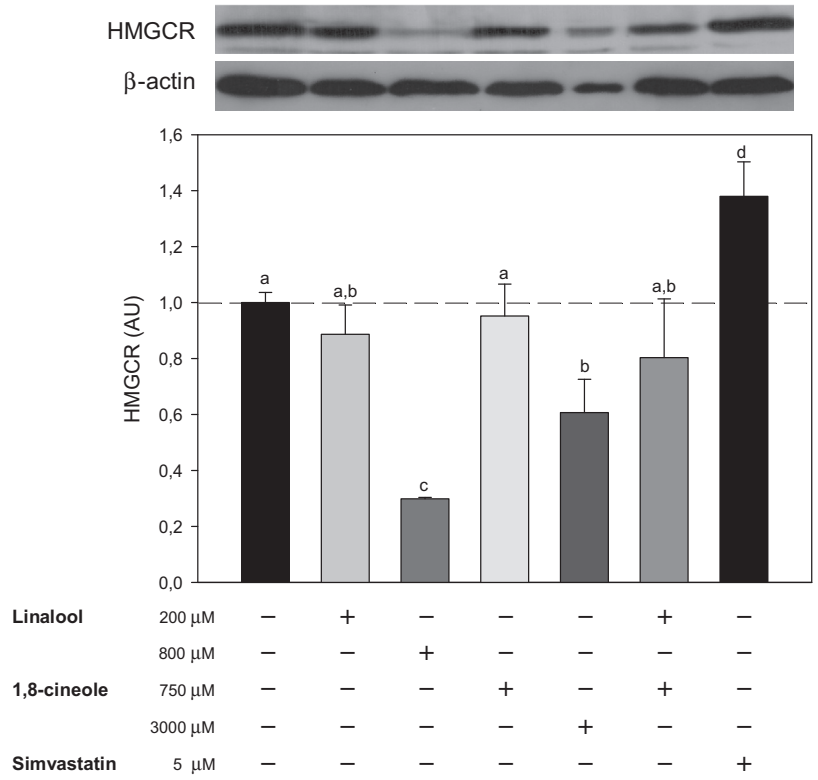

Fig. 7. Effect of linalool and 1,8-cineole on HMGCR protein levels in HepG2 cells. The cultured cells in exponential growth were treated with the isoprenoids at concentrations that inhibit both acetate incorporation into nonsaponifiable lipids and cholesterogenesis (800 µM linalool and 3.0 mM 1,8-cineole) or cholesterogenesis alone (200 µM linalool and 750 µM 1,8-cineole). Simvastatin at 5 µM was employed as positive control of increased HMGCR levels. The resulting bands were quantified by densitometric analysis and corrected for lane-loading differences on the basis of the relative actin-band intensities. The results are expressed as corrected band density relative to that of the control. Data are expressed as the means ± SD of three independent experiments; Means without the same letter are different ($p < 0.05$). AU, arbitrary units.

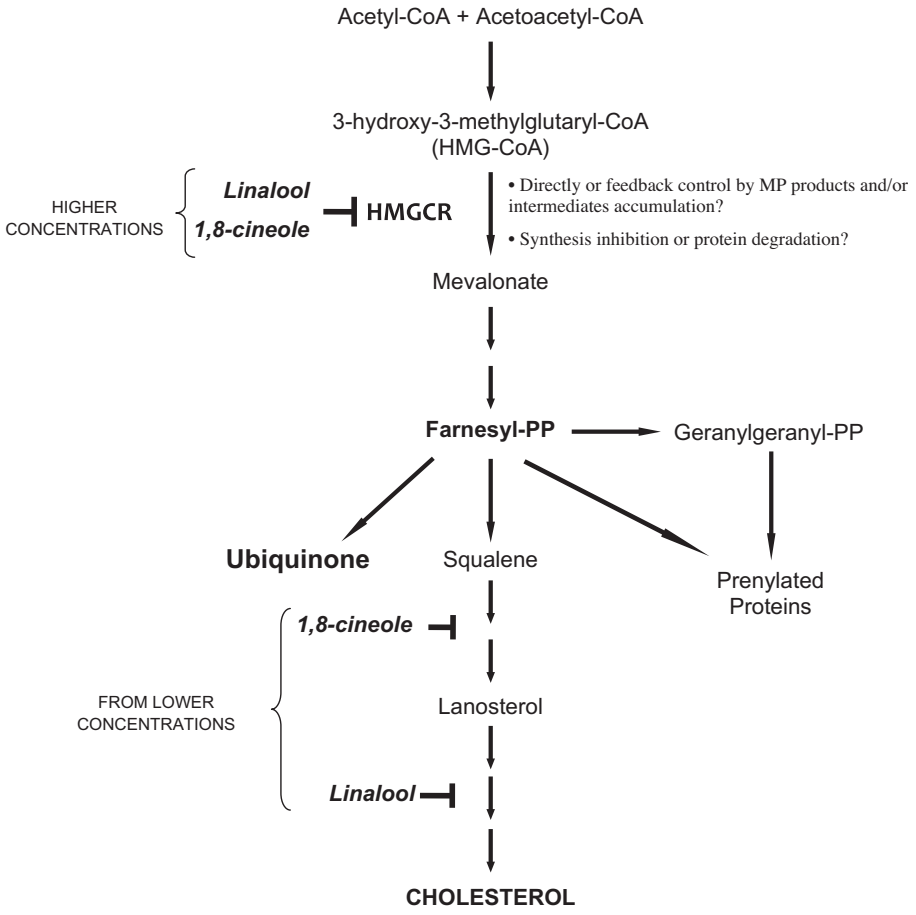

Fig. 8. Proposed model for regulation of the mevalonate pathway by linalool and 1,8-cineole in HepG2 cells. With low doses of linalool or 1,8-cineole an inhibition in cholesterol synthesis is produced. Whereas linalool generates an accumulation of lanosterol, 1,8-cineole causes a decline of this intermediate. Both isoprenoids evoke an increment in ubiquinone levels. Higher doses of linalool or 1,8-cineole reduce HMGCoA-reductase levels to produce a marked inhibition of cholesterogenesis without affecting cell proliferation.

in combination with monoterpenes. Even though additional combinations tested in this work confirmed the synergistic interaction between monoterpenes and simvastatin, additional concentrations and conditions should still be investigated to find the appropriate combinations for such a purpose.

Based on the results obtained in this work, we propose the model for MP regulation by linalool and 1,8-cineole summarized in Fig. 8.

Our data contribute to a better understanding of the action of the components of essential oils and their combination with statins in targeting a complex metabolic pathway and suggest that the use of those oils alone and in combination with the statins could provide significant health benefits in the treatment of cancer and/or cardiovascular diseases.

Conflict of interest statement

The authors declare no conflict of interest.

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